MECKEL'S CARTILAGE IN XENOPUS LAEVIS DURING METAMORPHOSIS

A study of the morphological and cellular changes taking place in Meckel's cartilage during metamorphosis in *Xenopus laevis* (Daudin).

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Handbuch

der

menſchlichen Anatomie

von

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Vierter Band.

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des Fötus.

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1820.

DECLARATION

I declare that this Thesis has been composed by myself, and that the work undertaken, and described therein, is my own.

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CONTENTS

LIST OF ABBREVIATIONS

C	Centigrade
cm	centimetre
CNP30	stabilised mixture of 1 1 1 - trichloroethane and tetrachloroethylene (Solvent used as a substitute for benzene)
g	gram
i.u.	International Units
L	Litre
mg	milligram
ml	millilitre
um	micrometre
MS 222	tricaine methane sulphonate
NF	Nieuwkoop and Faber

ABSTRACT

This thesis presents an analysis of the normal pattern of changes which occur in Meckel's cartilage in *Xenopus laevis* during metamorphosis. The investigation has concentrated on morphological as well as microscopic and ultrastructural changes which take place. A method for the preparation of the very fragile premetamorphic cartilage for electron microscopy has been developed. Some of the changes occurring in the cartilage were quantified using stereological techniques.

Appearance

During metamorphosis Meckel's cartilage changes from a shallowly curved bar to a deeper "U" shape. During this alteration, the cartilage changes from a structure with little intercellular matrix to a tissue in which both matrix and cellular components have increased considerably.

Cell population

During the early part of metamorphic climax there is a slow increase in the number of cells. A dramatic increase in the cell population occurs in the middle of the period, followed by a levelling off in the rate of cell divisions towards the end. Metamorphic climax has therefore been described in these three phases in this study.

These changes have been quantified by carrying out counts on the whole cartilage and methods of analysing such counts have been discussed.

Matrix production

The increase in the amount of matrix present is considerable, and largely accounts for the different appearances before and after metamorphic climax. This increase occurs in the later phase of climax, after the phase of maximal cell division has taken place.

This change has been quantified using a point counting technique, and the analysis of data from such techniques has been described.

Sites of cell division

The areas where most cell divisions are occurring during metamorphic climax appear to be mainly in the immediate subperichondrial zones of the tissue.

This has been investigated by examining sections for mitotic figures and by treating tadpoles during the period of maximal cell division with colchicine. A suitable method of using the drug for stathmokinesis during metamorphic climax has been described.

In addition to this investigation of normal metamorphic

changes, a preliminary study of the effect on Meckel's cartilage of exposing tadpoles to thyroid hormone at different times during metamorphic climax has also been undertaken, indicating that the hormone appears to act at a specific time during climax.

At attempt has been made to correlate the changes of shape and internal architecture of the cartilage with the changing feeding habit of the animals.

INTRODUCTION

This thesis presents a study of the changes taking place in some amphibian cartilages at metamorphosis. As a model, Meckel's cartilage in Xenopus laevis was selected, as it is a relatively small and discrete piece of tissue, unlike the components of the chondrocranium. Therefore the tissue as a whole, rather than samples of it, could be studied.

1 General Biology

The South African clawed frogs, genus Xenopus (family Pipidae) are found in many temperate regions of South and West Africa, especially Malawi and Zimbabwe. (Deuchar 1975). The most abundant species is Xenopus laevis (Daudin) which is divided into a number of subspecies on the basis of variations in body size and colour. For this study, a strain of animals bred under laboratory conditions for many generations was used (referred to in the text as Xenopus).

The family Pipidae comprises a primitive group of frogs (the Aglossa) which retain some fish-like characteristics, such as the absence of eye-lids. All the species are entirely aquatic, Xenopus living mainly in ditches, marshes and slow streams in veldt-type country. This mode of life is associated with a dorso-ventrally flattened body, with the long muscular hind limbs splayed out sideways. These limbs terminate in long toes which are fully webbed for rapid propulsion through the water. The medial three toes bear short, stout black claws, to

which Cochrane (1961) has ascribed a number of functions including stirring up mud in search of food, or for protection from predators.

The dorsal aspect of the animal is a grey-green in colour, with a very variable darker mottled pattern (Fig 1). This colouration may vary from a very pale green-brown to almost black depending on the animal's surroundings. Following anaesthesia with MS 222, (see Section I) the colouration becomes extremely pale, normal colour only being restored after a period of some hours. The animal's colouration also varies with the temperature of their environment, being darker at lower temperatures. The ventral surface is a pale cream in colour, often with a number of pale grey spots. The animal has a smooth, very slimy skin, making handling difficult, the glands producing this secretion becoming more active when the animals are disturbed. (Müller, 1976).

The adults are voracious feeders, living on almost any animal matter, alive or dead, the hind limb being used to tear up the food, and the forelimbs assisting to pack it into the mouth. The curious digits on the forelimbs, Leutscher (1976) describes as having considerable tactile sensitivity which is of value in the search for food. Further details of the natural history and general biology of Xenopus can be found in Beddard (1894), Bles (1905), Dreyer (1913, 1914), Deanesly & Parkes (1945), Weisz (1945 a, b, c), Nieuwkoop & Faber (1956), Brown (1970), Deuchar (1975).

2 Development

Like most amphibia, the development of Xenopus prior to metamorphosis takes place in two definitive periods (Goin & Goin 1971), the embryonic period before the hatching of the egg, and the free-swimming tadpole stage when the structure of the animal is quite distinct from the adult form. This stage lasts until metamorphosis, when the adult body form is attained, dramatic changes taking place within the animal very rapidly. Adult body structure having been attained, growth is fairly rapid for 18-24 months, when sexual maturity is reached. Growth continues beyond this time, in the male for up to 4 years (Deuchar 1975), while the females appear to continue growth throughout life, quite considerable size being attained in old specimens, up to 150g having been recorded (Deuchar 1975). A lifespan of 15 years has been recorded for captive specimens (Goin & Goin 1971). The lifespan in the wild is unknown, but presumably is much shorter than in such sheltered specimens.

Goin & Goin (1971) divide the Anuran tadpoles into four types, depending on the position and number of the spiracles and opercular chambers, and the form of the mouthparts. The tadpole of Xenopus is classified as Type I, as it possesses two opercular chambers, and lacks horny mouthparts. The tadpole diet consists of suspended microscopic particles which are filtered from the water by a mechanism supported by the branchial

cartilages. Such a feeding habit may be associated with the lack of keratinised mouthparts, which are found in most other Anuran genera.

The development of the tadpole is summarised in the Normal Table of Nieuwkoop & Faber (1956), which describes 66 Stages from fertilisation of the egg to the attainment of adult body form. These Stages are referred to in this study by number, and the abbreviation NF.

3 Metamorphosis

Metamorphosis is defined as a change of form particularly the marked change which some animals undergo in the course of development. Most amphibians undergo such changes, the transformation from tadpole to young adult involving not only alterations to external features, but also profound internal reorganisation involving many body systems.

Metamorphic climax is defined as the period of most rapid change between the final form of the tadpole and the attainment of adult body structure in miniature, and occupies Stages 58 to 66NF (Sedra and Michael, 1957) occurring between Day 44 and Day 58 post-fertilisation in animals maintained at 25°C (Table 1). The principal features of this period are summarised below (vide Normal Table of Xenopus laevis Nieuwkoop & Faber, 1956).

TABLE 1

NF Stage	Days post fertilisation
55	± 32
56	38
57	41
58	44
59	45
60	46
61	48
62	49
63	50
64	53
65	54
66	58

Time scale of development prior to and during metamorphic climax in tadpoles reared at 25°C.

a Limbs

The primordium of the hind limb first appears at Stage 46NF, and develops rapidly, the developing pelvic girdle and free portion of the limb being separable by Stage 50NF. Chondrification is completed in the femur by Stage 54NF, and ossification commences immediately thereafter, the distal parts of the limb following subsequently. The developing hindlimb is apparent externally from the beginning of its development; however the forelimb develops within a cavity, the atrium, and its primordium is first apparent at Stage 47NF. It is covered by a thin operculum during its early development, its eruption through this operculum at Stage 57-58NF indicating the commencement of metamorphic climax. Membranous ossification begins around the shaft of the humerus at Stage 55-57NF and a similar process is described in the femur by Fox & Irving (1950), endochondral ossification not commencing until the end of metamorphosis. The distal parts of the limb develop and ossify subsequently.

b Tail

One of the most striking features of anuran metamorphosis is the loss of the tail during metamorphic climax. The first indication of this in Xenopus is intercellular deposition of large quantities of melanin in the epidermis at the tip of the tail at Stage 58NF, which subsequently begins to atrophy. This deposition proceeds forwards rapidly, reaching

the base by Stage 62NF. From Stage 63NF degenerative changes are seen in the notochord and muscles. These changes proceed rapidly, the tail being lost by Stage 66NF.

c Skin

Below the entire epithelium the dermis thickens; however in some areas the epithelium undergoes metamorphosis, while in others the tadpole skin degenerates and shrivels. This metamorphic change occurs in both upper and lower jaws amongst other areas, and consists of intense proliferation in the basal layer, leading to the production of a new, thicker epithelium, the overlying tadpole epithelium sloughing off. Well developed adult epithelium is present along the edge of the lower jaw by Stage 59NF. As development of the metamorphosing skin areas progresses, the intervening areas of tadpole skin degenerate, until they meet all over the animal by Stage 65NF. In the strips of tadpole skin between the metamorphosing areas, the skin is connected to the underlying connective tissue surrounding the muscles by bands of loose fibrous tissue, these indicating the positions of the septa between the future lymph sacs. This pattern is defined by Stage 59-60NF.

d Alimentary canal

The first morphological changes associated with metamorphosis commence at Stage 59NF. At this Stage,

the lumen of the entire intestinal tract begins to narrow at the same time as the abdominal volume begins to decrease. This is followed at Stage 61-62NF by a considerable shortening of the tract. This reduction is continued until Stage 66NF. and is accompanied by an uncoiling of the intestine and a positional reorganisation of the abdominal contents from the tadpole to the adult situation. The lumen of the stomach widens at Stage 61-62NF, at the same time as a considerable increase in length occurs.

e Mouth and Pharynx

During metamorphic climax profound changes occur in these regions. The first row of tooth germs appears on the upper jaw at Stage 55NF, no teeth developing in the lower jaw. A lip fold develops along both upper and lower jaws at Stage 57NF, that in the upper jaw being larger. At Stage 62NF a second fold develops behind the teeth in the upper jaw, the lower jaw occluding between these folds. The lower jaw articulation moves caudally relative to the rostral end of the tadpole, resulting in an elongation of the mouth slit at Stage 61NF, the mouth opening migrating ventrally on the head as metamorphosis progresses. The branchial clefts begin closure at Stage 57NF, and are represented only by narrow grooves by Stage 64NF. A primitive tongue is indicated in the floor of the mouth by two longitudinal folds, and develops a short free tip at Stage 65NF.

f Head and lower jaw

One of the features of metamorphosis in Xenopus is the striking reduction in head size (Fig 2) with associated changes in the chondrocranium and lower jaw, which presents a quite different shape before and after metamorphosis (Fig 3).

The cartilages of the first branchial arch were first described by Johann Friedrich Meckel (1781-1833, Professor of Anatomy and Surgery in Halle) in 1820 and are named after him. He stated that these cartilages were present in fish, amphibia and birds, as well as in the human fetus. The paired Meckel's cartilages together with the unpaired inferior labial cartilage form the lower jaw in Xenopus. The primordia of the Meckel's cartilages appear as mesodermal condensations at Stage 40-41NF, and chondrification commences at Stage 42-43NF. The inferior labial cartilage first appears at Stage 43NF, and is cartilaginous by Stage 46NF.

The mandibular musculature begins to differentiate at Stage 40-41NF, and the tadpole muscle pattern is fully developed by Stage 55NF.

At this stage, the first evidence of developing bone in the lower jaw apears as a mesodermal condensation on the medial surface of the developing Meckel's cartilages, which is the primordium of the goniale.

Immediately prior to the commencement of metamorphic climax (Stage 57NF) the lower jaw of the tadpole consists of a single curved bar of cartilage, which Sedra & Michael (1957) describe as being composed of the two Meckel's cartilages, fused into a single structure through the median inferior labial cartilage. This complete structure is hereafter referred to as "Meckel's cartilage". Its shape is that of a shallowly curved bar, convex anteriorly (Fig 4). The originally separate inferior labial cartilage is curved downwards and the lines where fusion is occurring are still evident (Paterson,1939, Sedra & Michael, 1957, Shaw, 1982).

During metamorphic climax ossification is commencing in the upper and lower jaws. In the lower jaw, the primordium of the goniale and dentale commence membranous ossification at Stage 59-60NF around Meckel's cartilage, which persists as a discrete entity. How long it remains in the adult frog has not been ascertained, but it is present in its entirety up to the end of metamorphic climax (Paterson, 1939,Sedra & Michael,1957). By this time (Stage 66NF) Meckel's cartilage is becoming increasingly surrounded by the developing bone of the dentale and goniale. The cartilage remains a separate structure, the bones developing entirely by membranous ossification. By this means, Meckel's cartilage becomes progressively enveloped by bone,

remaining uncovered in the midline and at its articulation with the quadrate area of the base of the chondrocranium (Sedra & Michael 1957). However, the shape of the cartilage bar changes, due to a considerable increase in antero-posterior length accompanied by a decrease in width resulting in a much deeper "U" shaped curve (Shaw, 1982) (Fig 5). The plasticity of the immature bones developing as discrete structures around the cartilage enables them easily to accommodate these changes.

The tadpole musculature has been altered to the adult pattern by the end of Stage 66NF.

4 Cartilage in amphibians

The earliest evidence of primitive amphibians in the fossil record appears in the late Devonian rocks of Greenland, some 365 million years ago (Romer, 1941, Romer, 1959, Stirton, 1959, Young, 1981). The anurans first appeared during the late Triassic period around 220 million years ago (Beneš, 1979) and the genus Xenopus emerged around 20 million years ago in Africa, south of the Sahara (J P Shaw, personal communication).

The palaeontological origin of cartilage as a tissue is not known, partly because uncalcified tissues rarely leave any fossil evidence, but it is certainly much older than the earliest amphibians, having been present in vertebrates since their origin more than 500 million years ago, preceding bone as an endoskeletal, though not an exoskeletal tissue (Moss & Moss-Salentijn, 1983).

Although the literature on mammalian and human cartilage is very extensive, non-mammalian cartilage has received little attention in the last 50 years. Lubosch (1927) and Schaffer (1930) describe both invertebrate and vertebrate cartilages, though the latter did not accept that true cartilage exists in the invertebrates. However, Person and Philpott (1963, 1967, 1969) conclude that cartilage does occur in invertebrates, though with significant differences in structure and biochemistry from the vertebrate tissue.

Studies on amphibian cartilage have been carried out on adult animals especially concentrating on long bone formation (Haines, 1942, Fox & Irving 1950 a, Joyce & Cohen 1970, Dickson 1978, 1982) and fracture repair (Pritchard and Ruzicka, 1950) but a careful search of the literature has not revealed any work on either the structure of the tissue in the tadpole or on the changes occurring within it during metamorphosis.

The changes in the gross morphology of the cartilage of the head of Xenopus during metamorphosis have been described (Paterson 1939, Sedra & Michael 1957, Shaw 1982) and similar studies have been undertaken on the jaws of Bufo regularis (Sedra 1949) and Rana temporaria (Pusey 1938). However, no attempt has been made to study the striking changes in the internal architecture of the cartilage in Xenopus taking place in some of them at the same time. It was considered that Meckel's

cartilage could afford a suitable model for the study of these alterations.

The structure of the cells and matrix has been studied at light and electron microscope level, and a qualitative description of the changes occurring during metamorphic climax is presented in Section II.

Section III presents an attempt to quantify the change in cell number, and the change in matrix volume fraction is assessed in Section IV.

Having demonstrated that a considerable increase in cell numbers occurs quite rapidly during metamorphic climax, in Section V the stathmokinetic effect of the drug Colchicine was used to identify the sites at which this cell division was occurring. A suitable dose level of the drug appropriate to the tadpole's stage of development is described.

At attempt was made in Section VI to study the effect of thyroid hormone on the normal development of Meckel's cartilage by exposing tadpoles at various stages of development to this hormone.

Although metamorphic climax occupies Stages 58-66NF, this study has included Stage 57NF, as this Stage is described as pro-metamorphosis by Nieuwkoop and Faber (1956) and respresents the final structure of the tadpole.

SECTION I MATERIALS AND METHODS

A MATERIALS

1 Breeding Stock

Three large adult males and females were kept as breeding pairs. These animals were obtained from Xenopus Ltd.* The tadpoles used for the work described in this thesis were reared from eggs obtained from these animals in the laboratory by the author. The breeding adults were supplied as laboratory reared stock.

2 Sections II, III and IV

40 tadpoles from the same batch of eggs were used for the quantitative studies.

15 tadpoles from the same batch of eggs were used for orientation and ultrastructural examination.

3 Sections V and VI

30 tadpoles from the same batch of eggs were used as described in the appropriate Section.

(Section V also involved re-examination of some of the animals used for Sections II, III and IV).

B METHODS

1 Keeping of Adult Xenopus

Although the work described in this study was carried out on tadpoles during metamorphic climax, three pairs of large adult animals were maintained as breeding stock.

*Holmes Dale Nursery, South Nutfield, Surrey.

These animals were not used in any part of the experimental programme, and their husbandry was therefore divided into two phases, breeding (including preparation) and resting. Each pair was bred from at a maximum rate of once every three months.

a Resting phase husbandry

During this phase, the pair of animals was kept in a plastic aquarium, with close fitting wire mesh lid containing 10L of water at room temperature, which varied between 20 and 25°C. All water used for keeping adult frogs and rearing tadpoles was dechlorinated as recommended by the British Herpetological Society (1978). This was achieved by allowing hot water to stand in basins overnight, before filling the tanks and introducing the animals (Müller, 1976). Natural daylight in the animal room illuminated the aquaria, supplemented by fluorescent lighting for 6 hours during the day. The animals were fed with strips of raw liver on Mondays and Thursdays. The following day the animals were transferred by net to a tank of fresh water.

Despite the animals being described as normally nocturnal, (British Herpetological Society, 1978) food was accepted much more readily if the animal room was brightly lit on the day of feeding.

b The breeding phase (including preparation)

One month before use, a pair of animals was transferred to an aquarium containing 20L of water, dechlorinated and heated to 25°C. Feeding continued as during the resting phase, the animals being transferred to the smaller aquarium used during the resting phase, to avoid excessive fouling of the water in the large aquarium. The feeding aquarium was filled with 10L of water heated to 25°C and the animals returned to the main aquarium later in the afternoon.

When the animals were in breeding condition, the female's cloacal labia were reddened and enlarged, and the male showed well-developed rough dark nuptial pads on the inner aspect of the "hands", fingers and forearms. The animals came into breeding condition after one to three weeks in the heated water of the large aquarium. Injection of the males with a serum gonadotrophic hormone to bring them into breeding condition, as described by Shaw (1982), was not found to be necessary.

Mating and egg-laying can be induced artificially at any time of year in animals in suitable condition. For this, a chorionic gonadotrophic hormone preparation is injected into both male and female animals. The hormone preparation used for breeding was "Pregnyl"*, supplied by Xenopus Ltd.

*Organon Laboratories Ltd., Surrey.

The male and female having been brought to breeding condition as described above, both were injected with a solution of 500 i.u. of Pregnyl dissolved in 1 ml of sterile water. The male was given a single injection of 0.4 ml at 09.30 hours, while the female was injected with 0.3 ml at 09.30 hours, with a subsequent injection of 0.3 ml at 16.00 hours. It was found convenient and safer for the animals to anaesthetise them prior to injection, on account of the difficulty of handling such active and slippery creatures. The technique for anaesthetising Xenopus is as follows.

c Technique for anaesthetising adult Xenopus

The anaesthetic agent used was Tricaine methane sulphonate (MS 222)* supplied by Xenopus Ltd. The adults were anaesthetised by immersion in a tank containing 2L of water resulting in a depth of about 2.5 cm, into which was dissolved 3g of MS 222. This resulted in adequate anaesthesia in 20 minutes for safe handling. This slightly lower concentration of MS 222 (1.5g per litre) than that usually quoted (2g per litre) (Kaplan, 1969, Shaw, 1982) was found to result in adequate anaesthesia, with little if any subsequent sloughing of the outer layers of the skin.

To administer the injection to the anaesthetised animal, it is wrapped in a wet towel in such a way that the

*Sandoz Products Ltd., London

posterior half of the body and one hind limb are uncovered. The hypodermic needle is passed through the skin of the limb just lateral to the dorsal rows of lateral line organs. The needle is then advanced along the thigh and into the buttock towards the dorsal lymph sac where the solution is deposited. A slight resistance can be felt to be overcome as this structure is entered, which lies in the midline, dorsal to the cloacal opening. It was found to be important to keep the needle just subcutaneous, and if this was achieved for the full distance of penetration to the lymph sac, bleeding could be completely prevented. It was felt that this care taken to avoid bleeding was an important factor in the welfare of the animals, during the breeding phase. The animals were then returned to a tank of shallow water (2.5 cm) and carefully observed until normal breathing and mobility were recovered, usually five to ten minutes later. They were then returned to their aquarium. In the case of the female, the injection given at 16.00 hours approached the dorsal lymph sac from the other leg.

The injected animals were left undisturbed overnight, during which time amplexus took place and the eggs were laid. The adults were removed to another aquarium early the following morning, as the eggs were likely to be eaten by the adults, and those eggs that survived were

liable to be damaged by the agitation of the water caused by the powerful swimming movements of the adults. The eggs were left to develop in the breeding aquarium.

2 Care of the tadpoles

The tadpoles were kept throughout their development up to and during metamorphic climax at 25°C. At this temperature, development is quite rapid, the tadpoles hatching from the eggs during day 2 and attaching themselves to any solid object, or even the surface meniscus of the water, by threads of mucus. They remain motionless, attached in this way, for approximately 48 hours, commencing free swimming in the characteristic head downwards posture thereafter. Feeding began on the third day after hatching.

The tadpoles are filter feeders, as described in the Introduction, and were therefore fed on a suspension of powdered nettles*. This was found to be more satisfactory than commercially available tadpole food, which was apt to become mouldy after only a short time in storage. Producing a suspension of the nettle powder in water was facilitated by immersion of the powder overnight before use, as the very dry powder does not mix well with water. The animals were fed at 10.00 hours each day. Maintaining the cleanliness of the water in the tadpole aquarium required care, as any handling of the animals, if not actually physically damaging resulted in a delay in normal development, rendering the

*D Napier & Sons, Edinburgh

affected animals unsuitable for this study. However, by carefully syphoning off most of the unclean water, and replacing it with preheated, dechlorinated tapwater once per week, a healthy development of the tadpoles could be assured.

The installation of an aquarium filtration system consisting of an internal corner filter* powered by a 'Hy-Flo' pump* also assisted in maintaining the cleanliness of the water in the aquarium and thereby considerably reduced handling of the animals, helping to ensure a normal growth pattern.

The tadpoles were staged throughout development according to the Normal Table of Xenopus laevis (Nieuwkoop & Faber, 1956) which divides the development up to the completion of metamorphosis into 66 Stages. Metamorphic climax occupies Stages 58-66; for normally developing tadpoles days $\pm$ 44 to $\pm$ 54. During this period, food intake is much reduced, though not stopped, as described by Nieuwkoop & Faber (1956) and Shaw (1982). After metamorphosis the feeding changes to the carnivorous habit.

3 Killing the tadpoles

Animals that had reached the desired Stage were anaesthetised in a 500 ml solution of MS 222 at a concentration of 0.2g/L. As soon as the animals were immobile they were

*Griffen & George, Birmingham

killed by immediate immersion in various fixatives.

4 Preparation of whole mounts of lower jaws

The technique described below is a modification of that described by Dingerkus and Uhler (1977) for the differential staining of bone and cartilage following enzymatic clearing. The modifications were adopted because of the small size of the specimens.

a) The lower jaws were removed and fixed in 10% formalin for 24 hours, following which they were washed in distilled water for a further 24 hours. Skinning was not found to be necessary.

b) The specimens were then stained in a solution of 10mg alcian blue, 20ml glacial acetic acid and 80ml 95% ethyl alcohol for 24 hours.

c) The specimens were then returned to distilled water for 1 hour through 1 hour changes of a graded series of alcohols.

d) Clearing was achieved by placing the specimens in a solution of 1g of trypsin (beef - activity 0.5 Anson units/g), 30ml aqueous sodium borate and 70ml distilled water for 24-48 hours. Two days was found to be adequate for complete clearing.

e) The specimens were then macerated in 20ml of 0.5% aqueous potassium hydroxide, to which 5 drops of alizarin red S was added, for 24 hours, and subsequently washed for 1 hour in distilled water.

f) The jaws were then transferred to 100% glycerin, via a graded series of glycerin and water solutions, and stored in 100% glycerin.

This technique has been used to provide illustrations of the gross appearance of the cartilage, in the Introduction and Section VI of this study. The appropriate figures are identified by the caption "Cleared Whole Mount".

5 Preparation for light microscopy

a) The lower jaw was removed and fixed for 24 hours in Bouin's solution, and then washed for 1 hour in distilled water.

b) The specimen was then dehydrated using a graded series of alcohols, remaining in absolute alcohol for 24 hours.

c) Clearing was begun by placing the specimen in a solution of equal parts absolute alcohol and methyl salicylate for 1 hour, and completed by immersion in 100% methyl salicylate for a further 24 hours.

d) The tissue was then double embedded in celloidin and wax, by immersion for 24 hours in a solution of 0.5g of celloidin per 50ml of methyl salicylate, followed by 3 x ½ hour changes of CNP 30, and impregnation and embedding in molten paraffin wax. After embedding, the wax block was rapidly cooled under running water.

e) The block was then trimmed and serially sectioned at a thickness of 10µm on a Leitz rotary microtome, in the horizontal plane. The sections were stained with Heidenhain's iron haematoxylin using the technique described by Cullen (1979), to facilitate nuclear counting and morphometric analysis.

Lower jaws from animals at Stages 57, 60, 63 and 66NF were also sectioned transversely to assist in selection of sampling site and orientation of material for electron microscopy, and to complement the horizontally sectioned material in the description of the ossification occuring around the cartilage.

6 Preparation for electron microscopy

The problems of fixing this tissue especially at the early Stages are considerable, and a careful search of the literature has not revealed techniques described previously which are suitable for immature amphibian cartilage.

Dickson (1978) working with femoral cartilage from adult Rana obtained satisfactory fixation with 3.125% glutaraldehyde in 0.1M cacodylate buffer. Working on growth cartilages in the salamander, he found that the addition of magnesium or calcium ions improved the result (personal communication). However, attempts to fix immature Xenopus cartilage using these techniques resulted in extensive cellular disruption with fragmentation of the cell membrane and loss of intracellular organelles (Fig 6).

Jayatilaka (1978) obtained satisfactory fixation of pre-metamorphic thyroid tissue in Xenopus using 4% paraformaldehyde, 0.5% glutaraldehyde and 0.01% calcium chloride in 0.1M cacodylate buffer (pH 7.37) with post-fixation in osmium tetroxide. However, this technique also resulted in disruption of cell membranes and poor fixation of the intra-cellular organelles, especially mitochondria (Fig 7).

Dickson (1982) compared the results of fixation in glutaraldehyde and glutaraldehyde-formaldehyde mixtures, using cacodylate and phosphate buffers on the femoral growth cartilage in adult Rana. None of these methods resulted in any improvement in the fixation of immature Xenopus cartilage.

Sprinz and Stockwell (1976) used 1% osmium tetroxide dissolved in water to fix rabbit articular cartilage.

Based on their work, an attempt was made to fix tadpole cartilage directly in osmium, and the following techniques were tried.

a) Osmium dissolved in frog Ringer solution.
This technique resulted in better fixation of the cell membranes, but the cytoplasmic material was extremely diffuse, and the mitochondria grossly swollen, with destruction of their cristae (Fig 8).

b) Osmium dissolved in water with the addition of 0.02% calcium chloride.
This resulted in rather a similar appearance, the membranes being satisfactorily fixed, but the cytoplasm represented as diffuse fibrillar material, and the mitochondria being severely swollen (Figs 9,10).

As the damage resulting from techniques a) and b) above appeared to be osmotic, it was decided to attempt to fix the tissue in osmium dissolved in water with no additional ions, and this technique gave satisfactory results, the cell membranes were well fixed, the cytoplasmic material not fragmented, the mitochondria satisfactory and an endoplasmic reticulum system was preserved (vide Figs 35 and 36).

Technique

a) The animals were anaesthetised with MS 222 and killed by decapitation. The lower jaw was removed, cut in half and each part immediately immersed in 1% osmium tetroxide dissolved in water.

b) The specimens were then trimmed of excess tissue, dehydrated through a series of alcohols and embedded in araldite.

c) Following examination of 1µm sections stained with toluidine blue for orietation purposes, thin sections were cut at 80nm on a Reichert OMU3 ultramicrotome using a glass knife.

d) The sections were collected on copper grids, stained with 0.1% lead citrate and counterstained with a saturated solution of uranyl acetate in 50% alcohol.

e) The tissue was then examined in a Philips 301 transmission electron microscope at 60KV.

SECTION II MICROSCOPIC APPEARANCE AND ULTRASTRUCTURE

Cartilage in amphibians before metamorphosis is strikingly different in appearance from that of the mammalian tissue, and some cartilages, including Meckel's, undergo a radical change during metamorphic climax.

A LIGHT MICROSCOPY

1 Appearance

Prior to the onset of metamorphic climax, at Stage 57NF, the tissue consists almost entirely of very large lacunae, 20-40μm in diameter, and irregular in shape (Fig 11). The amount of interlacunar matrix present is very small, forming a very thin boundary line between the lacunae, resulting in a characteristic net-like appearance. The perichondrium is very thin, nowhere more than 1 or 2 cells thick, with the surrounding epithelium lying close against it along much of the length of the cartilage. At this Stage, Meckel's cartilage is quite similar in appearance to the ceratohyal cartilages lying just caudal to it in the lower jaw, although the lacunae in the latter are larger (Fig 12).

At its articulation with the quadrate area of the chondrocranium the peripheral cells are more closely packed, and lie in much smaller lacunae, with more matrix present at the articular surface (Fig 13). The perichondrium appears to be continuous with a lax joint capsule.

The very tenuous nature of the tissues in the tadpole at this stage renders them very susceptible to damage during fixation and subsequent sectioning.

As the metamorphic changes proceed the lacunae become smaller and the cells more densely packed, though the amount of matrix surrounding them does not at first appear to increase markedly. Small amounts of bone are starting to form within the perichondrium by Stage 60NF, (Fig 14). The goniale on the medial side of Meckel's cartilage begins to ossify a little in advance of the dentale, developing on the lateral side of the cartilage. By now the epithelia are separated from the perichondrium by a well defined lamina propria. Where ossification is not taking place, the perichondrium is still rather thin, and the lacunae are now smaller (10-20μm in diameter), though still surrounded by only a thin rim of matrix.

From Stage 60NF on, the amount of bone increases slowly in thickness, and the matrix appears also to start a very slow increase leading to a gradual separation of the lacunae.

By Stage 63NF, there is rather more matrix present giving rise to a reduction in the size of the lacunae, although the cartilage still presents a "net-like" appearance (Fig 15).

By the end of metamorphic climax (Stage 66NF) the cartilage

has a quite different appearance from that of Stage 57NF (Fig 16).

The amount of matrix present has increased markedly and the lacunae are now separated, often in groups of two or three. The more peripheral cells and those near the articular ends have more prominent cytoplasm, less densely staining nuclei and are more closely packed together, the deeper cells mainly lying in larger lacunae. The bone around Meckel's cartilage is becoming thicker (Fig 17, 17a) but is absent in the midline area and the articular ends. The perichondrium is thin, but quite dense except at the articular end where it becomes thicker, again being continuous with a loose joint capsule. At the articulation there is now a very dense concentration of cells, on both joint surfaces (Fig 18), which is not evident in the earlier stages of development. Apart from this area of increased cell density, the density appears to be constant throughout the remainder of the cartilage.

It is interesting to note that these cellular and matrix changes are not occurring in the ceratohyal cartilages during this phase, which retain their premetamorphic characteristics (Fig 19).

2 Ossification

In the animals used for this study, ossification commenced

in the goniale during Stages 58 and 59NF on the medial side of Meckel's cartilage (Fig 14), and in the dentale during Stage 60NF, on the lateral aspect.

The amount of bone increased slowly until Stage 62NF, and thereafter rather more rapidly. Throughout metamorphic climax the goniale was about 40-50μm in thickness, the thickest area being on the postero-medial aspect of the cartilage (Fig 17a). The dentale remained a thin film of bone on the lateral side of the cartilage. The articular areas, and the area around the midline remained uncovered by the developing bones until the end of metamorphic climax (Stage 66NF).

No evidence of endochondral ossification was seen in Meckel's cartilage at any Stage up to the end of metamorphic climax.

B ELECTRON MICROSCOPY

1 Appearance

The tissues were examined ultrastructurally at four stages during metamorphosis:

a) Stage 57NF - prometamorphosis

b) Stage 60NF - during early metamorphosis

c) Stage 63NF - prior to the increase in the matrix

d) Stage 66NF - at the end of metamorphic climax

Tissue was removed from the cartilage bar by transverse sectioning. The flakes of bone ossifying around the cartilage were trimmed away in the later Stages, prior to thin sectioning.

The appearance at Stages 57 and 60NF was very similar and results from these two Stages were grouped together.

a) Early metamorphosis (Stages 57 and 60NF)

At this stage, the cells on the periphery of the cartilage are flattened, with their long axes parallel to the tissue surface (Fig 20). They exhibit a high nucleus to cytoplasm ratio, some granular endoplasmic reticulum and large mitochondria. The perinunclear cisternae are dilated and some ribosomes are present on the outer nuclear membrane. Many of the cells exhibit a well-defined Golgi complex close to the nuclear envelope (Fig 21, 22). Their processes are short and the lacunar matrix around each cell is not well

defined. This layer is only 1-2 cells thick, lying superficial to a zone of larger spheroidal cells. These cells also have a large nucleus with prominent perinuclear cisternae and an extensive granular endoplasmic reticulum is present. They have short processes and the lacunar matrix is somewhat better demarcated. Some of the matrix fibrils show aggregation into small bundles at the lacunar margins (Fig 23). The matrix contains large numbers of fine, randomly oriented fibrils interspersed with small matrix granules.

However, in the zones indicated in Figs 24a and b this transition from flattened to rounded cells is interrupted by an additional zone. Here, the cells are arranged in distinct columns, arranged perpendicular to the surface, just below the flattened peripheral cells (Figs 25, 26). The cell density in this area is high, and two cells are frequently seen lying in the same lacuna. The nuclear material is evenly distributed and one or both nucleoli often prominent. Mitochondria are numerous and the endoplasmic reticulum well developed, with often a well-defined Golgi region near the nucleus. The cells possess short, fine processes, confined within the lacuna and the interlacunar matrix is clearly distinguishable, forming a thin rim of more densely packed fibrils defining the territorial zone of each cell. In some situations the fibrils in the lacunar matrix appear to be aggregating to form such a rim,

separating two recently divided cells (Fig 27).

Deep to this proliferative zone the cells are of varied shape and occupy irregularly shaped zones of lacunar matrix (Fig 28). An extensive juxtanuclear Golgi area is associated with vacuoles, some of which appear to be opening into the lacunae, and may contain matrix components (Fig 28). The cells exhibit a prominent granular endoplasmic reticulum with dilated cisternae. The lacuna is a small area immediately surrounding each cell, which has a few short processes. The inter-lacunar matrix in some areas shows longer, irregularly arranged fibrils than the more superficial zones, interspersed with matrix granules (Fig 28).

In the deepest zone of the cartilage, interspersed with hypertrophic cells, are cells with a very large nucleus, and a cytoplasm apparently fragmenting, and filled with large areas of material rather similar to the lacunar matrix. The mitochondria are distended and there is little granular endoplasmic reticulum present. These apparently degenerating cells are found usually singly within the cartilage (Fig 29). Also within these areas are found single, rather palely staining cells with characteristically irregularly shaped nuclei, whose cytoplasm contains large numbers of fine fibrils lying mainly around the nucleus (Fig 30).

In many other sites, even amidst apparently proliferative cells, dense, irregular bodies, some of which appear to be membrane bound, are seen, usually near the junctions of areas of inter-lacunar matrix (Figs 31 and 32).

b) Stage 63NF (Before matrix increase)

The most noticeable difference in the cartilage by this Stage is that there is no longer any evidence of the cell columns seen earlier.

The peripheral cells are less flattened, being rather irregular in shape with short, fine processes. Most cells show an extensive granular endoplasmic reticulum, Golgi apparatus and numerous secretory vesicles filled with a moderately dense material. The lacunar matrix forms a narrow zone around the cells, and some condensation of fibrillar material is apparent in the inter-lacunar matrix with some orientation of the fibrils (Figs 33 and 34). This peripheral zone is now several cells thick and merges into the deeper cell layers gradually.

The deeper cells have a much more spheroidal shape (Fig 35) and usually a large nucleus, often showing one nucleolus. There is a very extensive granular endoplasmic reticulum, the cisternae of which are dilated and filled with material of moderate electron density (Fig 35). There is a large elaborate Golgi apparatus situated close to the nucleus (Fig 36).

The cells possess numerous short processes extending through the small area of the lacuna, which is usually clearly defined from the more dense interlacunar matrix, consisting of randomly oriented fibrils, interspersed with small matrix granules. Many of the cells possess a large number of vesicles containing amorphous material, some of which appear to be opening to the surface, and large numbers of irregularly shaped mitochondria, often concentrated at one end of the cell (Fig 36).

Some of the deeper cells were observed to have a single aberrant cilium (Fig 37). These cilia have a basal body which is one of the centrioles of the cell, and this is accompanied by a second one close by (Fig 38).

As in early metamorphosis, a number of solitary cells, apparently undergoing degenerative changes, and areas of cellular debris are seen at all levels in the cartilage.

(c) Stage 66NF

By the end of metamorphic climax, the most obvious feature of the cartilage is the increase in the amount of interlacunar matrix.

The peripheral zone of the cartilage now comprises more matrix, the cells being separated by large amounts of matrix with loosely aggregated fibrils (Fig 39).

The cells deep to this area are numerous, irregularly shaped, lying in well-defined small lacunae (Fig 40). They exhibit irregularly shaped nuclei, numerous mitochondria and quantities of granular endoplasmic reticulum, with frequently a prominent Golgi apparatus. The cell membrane has few fine processes, extending through the lacuna. The interlacunar matrix consists of irregularly arranged, closely aggregated fibrils, interspersed with matrix granules (Fig 41).

A feature of many cells in all zones within the cartilage is the presence of lysosomes (Figs 42, 43) not observed in cells during early metamorphosis or at Stage 63NF. The lysosomes usually have a narrow electron-lucent zone between the limiting membrane and the homogenous, moderately electrondense contents.

As in early metamorphosis and Stage 63NF, solitary, apparently degenerating cells are present in the tissue, mainly concentrated in the deepest areas (Fig 44).

C DISCUSSION

1 Cell Zones

The similarity between the cell zones in Meckel's cartilage during early metamorphosis and those described in the epiphyseal cartilage in Rana by Dickson (1982) is quite striking. This resemblance is interesting, as Meckel's cartilage in mammals does not show this similarity.

During early metamorphosis (Stages 57 and 60NF) the outermost layers of cartilage cells are flattened and their long axes are parallel to the tissue surface, which appears to be similar to the reserve zone described by Dickson (1982) found immediately subjacent to the articular cartilage.

The arrangement of the cells into columns below this reserve zone in the areas of the cartilage indicated in Fig 24a and b is similar to Dickson's (1982) description of the proliferative zone, although he does not present convincing photographic evidence for such a zone. This arrangement appears to be associated with the changing shape of the cartilage, particularly the increase in length occurring in this region.

The zone lying deeper within the cartilage, with rather irregularly shaped cells appears similar to the hypertrophic zone described by Dickson (1982), and the larger numbers of such cells later in metamorphic climax, exhibiting

considerable dilation of the granular endoplasmic reticulum is probably associated with the production of matrix occurring at this time.

2 Matrix

The appearance of the matrix even during prometamorphosis (Stage 57NF) is similar to that described by Dickson (1982) in the adult frog growth cartilage. During metamorphic climax, the amount of matrix present increases, and it becomes more densely fibrillar especially in the inter-lacunar area, but the fibrils and small granules do not alter markedly in size or appearance. Dickson (1982) described the small matrix granules in the adult frog as attached to the fibrils whereas in Xenopus these structures are also seen scattered in the matrix unattached to fibrils.

As the cartilage matures during metamorphic climax, the fibrils begin to condense and become orientated around the periphery of the lacunae.

Sheldon (1983) described the lacunar matrix in mammalian cartilage as containing few of the normal fibrils of cartilage matrix, and states that this is the result of maturation and polymerisation of newly synthesised matrix components taking place in this area. As the fibrillar material within the lacuna is also less dense in Xenopus it seems likely that a similar process is occurring in maturing amphibian cartilage.

3 Cilia

The single cilia observed on some of the deeper cells have been described by Scherft and Daems (1967) in embryonic mouse cartilage, by Stockwell (1971) in fetal sheep cartilage, by Stockwell and Meachim (1973) in human adult articular cartilage, and by Wilsman (1978) and Wilsman and Fletcher (1978) in adult and juvenile canine cartilage.

Such cilia have been reported in a wide variety of tissues, summarised by Fawcett (1981), but do not appear to have been previously recorded in amphibian cartilage. Using stereological techniques, Wilsman (1978) and Wilsman and Fletcher (1978) indicated that single cilia are a common feature of canine chondrocytes, probably each cell possessing one.

As described by Scherft and Daems (1967) these amphibian examples have an intimate relationship with the centrioles of the cell.

These structures are probably non-motile, and a number of functions have been attributed to them including sensory reception, none of which appears likely in a cartilage cell, and it is possible that they are merely anomalous rudimentary structures as suggested by Fawcett (1981), though Carr and Toner (1982) suggest that they may represent a normal aspect of centriolar potential.

4 Cell debris and degeneration

From the earliest Stage examined (57NF) cells exhibiting evidence of degeneration are seen, along with cell debris, mainly in the deeper zones of the cartilage. The degenerating cells are generally found singly, and exhibit either a cytoplasm which is fragmenting, distended mitochondria, and ruptured cell membrane, or are pale staining cells containing large numbers of fine fibrils.

In fetal rat cartilage, fine filaments have been described by Godman and Porter (1960) who described them lying just below the cell membrane, and speculated that such filaments were about to be exocytosed, to act as "seeding" agents for further fibril growth in the pericellular matrix, resulting in the mature matrix collagen fibres. However, more recent work by Barnett et al (1963), Meachim and Roy (1967) and Sprinz and Stockwell (1976) has indicated that this explanation is unlikely, and that such perinuclear accumulations of filaments, when present in large quantity are evidence of degenerative changes. The dense, irregular bodies seen in the matrix during early metamorphosis are similar to those described by Ghadially et al (1978) and Dickson (1982) and probably represent the granular debris of cells. These structures occurring during prometamorphosis (Stage 57NF) amidst apparently proliferating cells indicate that even in areas where cell division is occurring, cells are also dying.

Lipid accumulation, which is a feature of such changes in mammalian cartilage cells (Stockwell, 1979) does not appear to be so in the amphibian tissue during metamorphic climax.

5 Lysosomes

The presence of lysosomes has been described in the cells at the end of metamorphic climax (Stage 66NF). These organelles have been identified on morphological grounds in this study and their structure appears to be closely similar to those described by Daems and Van Rijssel (1961) in mouse liver cells, and those illustrated by Stockwell (1979) in rabbit articular cartilage. The presence of acid phosphatase activity has not been investigated, described by Stockwell (1979) as an essential criterion for distinguishing them from other vacuoles, and consequently only a tentative identification is possible.

Lysosomes in cartilage cells, in addition to their normal role in cell metabolism, are described as playing an important part in the local control of the matrix (Stockwell 1979). Dingle (1975) described exteriorisation of lysosomal enzymes by fusion of the lysosome with the cell membrane, followed by endocytosis of digested matrix components.

Such structures were not observed in the material prepared at earlier Stages, and it seems likely that this increase in lysosomal activity is concerned with turnover of matrix components associated with the changing shape of the cartilage (Shaw 1982).

SECTION III CHANGES IN CELL POPULATION DURING METAMORPHOSIS

A METHOD

1 Preparation of material

This study was carried out on 40 animals during prometamorphosis and metamorphic climax, Stages 57-66NF, 4 animals being used at each Stage.

Eggs were obtained, and the animals reared by the methods described in Section I. The tadpoles were allowed to develop to Stage 55NF, when all those reaching this stage on the same day were transferred to a separate aquarium.

This experimental group was examined and staged daily while swimming in the aquarium and animals reaching the same Stage on the same day were selected. In this way animals which appeared to be developing at a comparable rate were used.

The animals selected at each Stage were removed from the aquarium and killed. The staging carried out on the living animals was now confirmed by more detailed examination of criteria not easily assessed in the living tadpole. Only the 4 animals most closely conforming to each NF Stage were used.

The lower jaws were removed from these animals and processed for examination with the light microscope.

2 Technique for counting

The slides from each specimen were examined, and those showing parts of Meckel's cartilage were marked. Every fifth section was selected for analysis, and the nucleus was chosen as the unit to be counted.

Counting was carried out at a magnification of x 250 using a Leitz Laborlux 12 microscope fitted with a drawing tube attachment. This enabled a sheet of paper to be superimposed on the field of view. A felt-tip pen mounted in a holder incorporating a switch attached to an electronic counter* was then used to mark each nucleus on the paper. As the nucleus was marked, the switch activated the counter, registering each nucleus. A yellow pen was used, contrasting with the black-stained nucleus, and using this technique the double counting of nuclei could be avoided. The magnification factor of the drawing tube (x 1.25) ensured adequate separation of the dots.

Where the area of the section of Meckel's cartilage extended beyond the field of view on the microscope, after completing the count three prominent lacunae, together with the external contour of the cartilage, were outlined on the paper, the slide moved and the paper moved correspondingly. In this way, counting of the whole cartilage could be carried out accurately.

*Scientifica and Cook Electronics, London

3 RESULTS

The results of these counts are presented in Table III_1, together with the mean nuclear number for each Stage, and standard deviation.

C DISCUSSION

Many techniques are available for estimating cell populations, all developed from four basic methods:

1 Total count method
2 Systematic section sampling
3 Random section sampling, and
4 Sample and volume.

It was decided that for this study of changing cell numbers during metamorphosis a systematic section sampling technique would be most appropriate.

Total counts are taken as probably yielding the most accurate estimate of cell numbers, but studies comparing them with systematic section counts have indicated that the level of accuracy of the latter technique is high if an appropriate periodicity of count is used for the size of the structure.

Moatamed (1966) carried out total cell counts of the human inferior olivary nucleus and then compared these with systematic counts of all cells at periods from 2 to 300

TABLE III_1

Stage	A	B	C	D	Mean	Standard Deviation
57	37,265	36,950	34,235	38,005	36,613	± 1646
58	41,120	36,780	40,325	42,120	40,086	± 2323
59	44,220	42,835	46,905	47,930	45,472	± 2353
60	48,215	48,840	44,930	49,535	46,880	± 2039
61	57,340	56,290	55,365	52,210	55,301	± 2213
62	77,245	81,675	80,880	76,270	79,017	± 2659
63	80,530	76,450	80,610	82,480	80,017	± 2543
64	87,590	82,320	88,160	83,920	85,497	± 2831
65	93,555	97,565	91,535	86,335	92,247	± 4670
66	92,135	93,575	91,850	92,930	92,622	± 782

TOTAL NUCLEAR COUNTS

Uncorrected

(A period is the interval between the sections counted). A period of 5 was accurate to ± 0.5% while a period of 10 resulted in an accuracy of ± 1.3%. Radmanesh (1968), counting nucleoli in the human nucleus intercalatus, found that counting every 5th section gave an accuracy of ± 6% and every 10th ± 8.8%. Konigsmark et al (1969) reported counting neurons in every 10th section to be accurate to ± 5% and every 20th ± 10%. It seems likely that the variation in these figures depends on the variation in cell number between adjacent sections, and this in turn would be influenced by the shape of the structure being counted, and the regularity of the cell density within it. As the distribution of cells within Meckel's cartilage in Xenopus is irregular, and, because of its curving shape the number of nuclei present on adjacent sections can vary widely, a periodicity of 5 was selected. This corresponds with counts on the nucleus intercalatus, described by Konigsmark (1970) as a long thin structure where there is considerable variation in cell numbers between sections, rather comparable to Meckel's cartilage.

The random section method was rejected as Konigsmark et al (1969) found the error to be significantly greater than with systematic sampling.

The sample and volume method is a useful technique where the volume of the structure can be accurately measured,

but the estimation of volume from measurements on microscopic sections introduces a further source of error and so the method is unlikely to offer greater accuracy than the systematic section method.

Two further sources of error must be considered with all methods of nuclear or nucleolar counting, namely the problem of the accuracy and repeatability of the count, and the problem of the split-cell error.

a) Counting error

Duncan and Keyser (1936) repeating counts made by the other co-author, using an ocular grid and microscope directly, or counting on drawings, showed discrepancies of results of up to 10%. Gardener (1940), on recounts of sections, found a variation in total of up to 5%, the average being 1.6%. However, no indication is given of whether these recounts were by the same or a different individual, nor of the counting technique used. Ebbesson (1963), with the same individual repeating the counts, reported accuracy to within 2.8% while Moatamed (1966), with a second person repeating the count, found the variation to be within 2%, both these authors using a direct counting technique. Konigsmark (1970) reports an accuracy of usually within 3% and frequently 1%, but does not state who carried out the repeat counts.

It was therefore decided to carry out repeat counts on 20 randomly selected sections, using the counting technique described above. Only one person was involved in the counting, and so all repeat counts were carried out by the same individual. These results, together with the percentage variations are presented in Table III_2 and indicate an average counting error of 2.4%, according closely with the findings of other workers.

b) Split cell error

Any count of discrete bodies on sectional material raises the problem of a structure split between two sections being counted twice. This problem obviously increases the larger the object being counted. Agduhr (1941) first described this source of error in relation to earlier work involving cortical neuronal counts by Von Economo and Koskinas (1925). These authors presented their findings having made no allowance for overcounting due to split neurones. Agduhr then went on to derive a formula (1) to correct for this.

Formula 1 $$Ni = \frac{2t-d}{2t}$$

where Ni is the actual number of units in section i, ni is the number of units counted, t is the thickness of the section and d is the unit diameter.

Since Agduhr's paper, many authors have worked on this

TABLE III_2

SECTION	1st COUNT	2nd COUNT	% DISCREPANCY
58AG	551	574	4.1
65AN	839	851	1.4
58CK	229	219	4.3
65BM	1193	1176	1.4
65DY	236	226	4.2
58DCC	440	429	2.5
65EJ	1294	1279	1.1
58BO	105	98	6.6
59AE	218	221	1.3
60AP	481	477	0.8
61AF	760	771	1.4
62AW	338	344	1.7
63AQ	436	430	1.3
64AL	872	876	0.5
64BQ	364	355	2.4
59BN	304	291	4.2
59CP	349	344	1.4
59DR	374	380	1.5
60BE	341	350	2.6
60DI	228	221	3.0

MEAN PERCENTAGE DISCREPANCY = 2.4%

REPEAT COUNTS CARRIED OUT ON RANDOM SECTIONS TO DETERMINE COUNTING ERROR

(MAGNIFICATION x 250)

problem, mainly developing and refining Agduhr's basic formula.

Abercrombie (1946) particularly emphasised the importance of the size of the nucleus relative to the section thickness and derived Formula 2 using these parameters.

Formula 2 $Ni = ni\frac{t}{t+d}$

Konigsmark et al (1969) developed a formula (3) taking into account nuclear diameter and thickness, and also uncounted fragments of the structure under examination (in this paper, neurones).

Formula 3 $Ni = ni\frac{t}{t+2a}$

where $a = \sqrt{r^2 - (\frac{k}{2})^2}$ and r = the radius of the counted unit, and k = the maximum diameter of uncounted fragments.

It is difficult to determine a definitive figure for the largest diameter of uncounted fragments, as several factors are involved. Marrable (1962) considered theoretical methods for assessing a value for k, and quotes a figure of 1.45μm, but does not explain how this figure is arrived at. Konigsmark (1970) considering the limit of

resolution of the light microscope, derives a minimum possible value, and concludes that the theoretical limit for k must be 0.5μm (for an immersion objective of N.A. 1.0, 0.24μm).

However, the value of k also depends on the staining technique used and the efficiency of the optical system. Furthermore, Ebbesson and Tang (1965) showed that operator alertness was an important factor.

Assessment of k is therefore an arbitrary process, dependent on the specific conditions of the experiment. In the case of the present investigation, the size of the smallest nuclear fragments counted could not be accurately measured at the magnification used for counting (x 250), as the scale divisions of even a fine eyepiece graticule were more widely spaced than the diameter of the fragments, and therefore only an estimate of size was possible. This estimate was made as 1μm, as below this size not only are fragments difficult to visualise, but it becomes increasingly uncertain whether in fact a stained fragment is part of a nucleus.

In deriving their formulae, all the authors cited have worked on the assumption that the diameter of the structure being counted is less than the section thickness.

From the formulae quoted above, it is evident that an estimation of split cell error is based on the diameter

of the counted object, section thickness and an estimation of a value for k, therefore these parameters were measured on the material under study.

Ten nuclei at each Stage were measured using an eyepiece graticule previously calibrated against a stage micrometer at a magnification of x 1000 using a Leitz Laborlux 12 microscope. For the purpose of assessing the correction factor, the nuclei were assumed to be spherical.

The thickness of 20 sections (2 from each Stage) selected at random was measured by focussing on the top of the section, and repeating this on the bottom, and reading off the distance travelled by the stage on the graduated fine focus control, each scale interval corresponding to 2µm of travel on the microscope used (Leitz Laborlux 12). With care the nearest quarter divisions could be assessed, giving an accuracy of $\pm$ 0.5µm. These results are presented in Tables III_3 and III_4. From these figures, using Student's "t" test, described by Moroney (1969) as suitable for small samples, there appears to be no significant change in nuclear diameter from Stage 57 to Stage 66NF (Table III_3) and the section thickness, although greater than that indicated by the micrometer on the microtome is also similar for all Stages. Konigsmark (1970) states that the micrometer setting on microtomes is not a reliable guide to the actual section thickness, but it would be

TABLE III_3

DIAMETER μm

STAGE											Mean	Standard Deviation
57	5.5	6.0	6.0	6.0	6.5	5.5	6.0	6.5	6.0	6.0	6.5	± 0.33
58	6.0	6.0	6.5	6.0	6.5	5.5	6.0	6.0	6.0	6.0	6.0	± 0.28
59	6.0	5.5	6.0	6.5	5.5	6.0	5.5	6.0	6.0	5.5	5.9	± 0.33
60	6.0	5.5	6.0	5.5	6.0	6.0	6.0	5.5	6.0	6.0	5.9	± 0.24
61	6.0	5.5	5.5	6.0	6.0	6.0	6.5	6.0	6.5	6.0	6.0	± 0.33
62	6.5	6.0	6.0	6.0	6.0	6.5	5.5	6.0	6.0	6.0	6.0	± 0.28
63	6.0	6.5	6.0	6.0	5.5	6.0	6.5	5.5	6.0	6.5	6.0	± 0.37
64	5.5	6.0	6.5	6.0	6.5	6.0	5.5	6.5	6.0	5.5	6.0	± 0.41
65	6.0	6.0	6.0	6.5	6.0	5.5	6.0	5.5	6.5	6.5	6.1	± 0.37
66	6.5	6.0	6.0	5.5	6.0	6.0	6.0	6.0	6.0	6.5	6.1	± 0.28

MEASUREMENT OF NUCLEAR DIAMETER (x 1,000)

TABLE III_4

FOCUS AT TOP OF SECTION	FOCUS AT BOTTOM	SECTION THICKNESS (μm)
43	49	12
41	47.5	13
41	47	12
43	49	12
43	48.5	11
43	49	12
42	47.5	11
42	48	12
43	49	12
42	48	12
43	49	12
41	46.5	11
43	49	12
42	48.5	13
42	48	12
43	49	12
43	48.5	11
41	46.5	11
41	47	12
42	48.5	13

MEAN SECTION THICKNESS: 11.9μm
STANDARD DEVIATION: ± 0.64

MEASUREMENT OF SECTION THICKNESS (MAGNIFICATION x 400)

Micrometer setting on microtome: 10μm

expected that the actual thickness would vary around the micrometer reading, as described by Marengo (1944) who stated the error was around 10%. However, Table III_4 indicates that the section thickness may in fact be quite different from the micrometer reading (in this case, 20% greater) and is therefore an important parameter to measure if absolute quantification from such sections is to be reliable.

The estimated value for k is assumed to be constant for all Stages, as the material has been prepared in the same way, and the nuclear diameter does not change significantly. Substituting these figures in Formula 3 gives a correction factor of 0.67 for all Stages. The corrected figures for nuclear number, together with the means and standard deviations, are presented in Table III_5 and graphically in Fig 45.

The number of nuclei counted is assumed to indicate the number of cells present in the cartilage.

From these data, it would appear that, at the beginning of metamorphic climax, cell division is occurring at a slowly increasing rate until Stage 60NF. Between Stages 60 and 62NF, cell division is occurring rapidly, the rate decreasing again as the end of climax is approached.

TABLE III_5

Stage	A	B	C	D	Mean	Standard Deviation
57	24,968	24,757	22,937	25,463	24,531	± 1103
58	27,550	24,643	27,018	28,220	26,858	± 1556
59	29,627	28,699	31,426	32,113	30,466	± 1576
60	32,304	32,722	30,103	33,188	32,079	± 1366
61	38,419	37,714	37,095	34,981	37,052	± 1483
62	51,754	54,722	54,190	51,101	52,941	± 1782
63	53,955	51,222	54,009	55,262	53,612	± 1704
64	58,685	55,154	59,067	56,226	57,283	± 1897
65	62,682	65,369	61,328	57,844	61,806	± 3130
66	61,730	62,695	61,539	62,263	62,057	± 524

CORRECTED TOTAL NUCLEAR NUMBERS

Using Student's "t" test, the most significant changes in cell population take place between Stages 57 and 60NF ($p < 0.001$), 60 and 62NF ($p < 0.001$) and between 62 and 66NF ($p < 0.001$). The period of most rapid cell division (Stage 60-62NF) corresponds with the period of most rapid change of jaw shape, described by Shaw (1982). As this most rapid phase of cell division occurs in the middle of metamorphic climax it is possible, on the basis of changing cell population to divide the period of climax into three phases.

Phase I - Pre-division climax corresponding to Stages 57 to 60NF, represents a period of slowly increasing cell division.

Phase II - Division climax corresponding to Stages 60 to 62NF, represents a short period of intense cell division.

Phase III - Post-division climax corresponding to Stages 62 to 66NF, represents a gradual levelling off in the rate of cell division.

These three phases, together with the NF Stages and time scale in days are demonstrated graphically in Fig 46.

D CELL DENSITY BELOW THE ARTICULAR SURFACE

As there appears to be a change in the density of cells

beneath the articular surface during metamorphic climax (Figs 13, 18) it was decided to extend the quantification studies by a more detailed examination of this area.

1 Method

Sections were selected from all 4 animals at Stages 57, 60, 63 and 66NF showing the articulation of Meckel's cartilage with the quadrate. A further selection was made of sections which showed the maximum width of Meckel's cartilage, to avoid the artificially increased number of nuclei in tangential sections near the periphery of the cartilage.

Nuclear counts were made using a squared graticule at a magnification of x 400 superimposed on randomly selected sites, avoiding the sides of the cartilage sections. The number of nuclei or nuclear fragments lying in a rectangular area 80μm x 20μm placed with its long axis parallel to and abutting on the articular surface was recorded. This was repeated in successive areas, to a depth of 200μm below the surface, at right angles to it (parallel to the long axis of the cartilage). This was carried out 5 times on each specimen.

The same correction factor as derived for the total nuclear counts was applied.

2 Results

The corrected nuclear numbers for each level along with the mean and standard deviations are presented in Table III_6 and graphically in Fig 47.

It is evident from these data that the cell population beneath the articular surface undergoes marked change during metamorphic climax, to a depth of 140-160μm.

Between Stages 57 and 60NF the increase in cells present is solely in the superficial zone (0-20μm). After Stage 60NF, an increase in cell numbers in the two deeper zones (20-40μm and 40-60μm) becomes evident, while the cell population of the most superficial zone (0-20μm) continues to rise. Below the 140-160μm zone, the cell population shows no significant change during metamorphic climax (Student's "t" test).

3 Discussion

The cell distribution beneath the articular surface of Meckel's cartilage in Xenopus appears to be comparable with that described in cartilages in mammals, where the density is described as maximal at the surface, where the cells of the perichondrium are merging imperceptibly with those of the cartilage. This cell density continues to diminish within the cartilage until a plateau is reached (Maroudas, Stockwell, Nachemson and Urban, 1975). A

TABLE III_6

DEPTH \ ANIMAL		A	B	C	D	MEAN	STANDARD DEVIATION ±
57	0- 20 µm	42.2	35.5	26.8	36.2	35.2	6.3
	20- 40	12.7	13.4	12.1	12.1	12.6	0.6
	40- 60	7.4	10.7	7.4	10.7	9.0	1.9
	60- 80	6.0	4.0	4.0	5.4	4.0	1.0
	80-100	5.4	4.0	4.7	4.0	5.0	0.7
	100-120	2.0	2.0	4.7	4.0	3.2	1.4
	120-140	4.0	0.7	2.7	4.7	3.0	1.8
	140-160	4.0	2.7	3.4	4.0	3.5	0.6
	160-180	3.4	4.7	4.7	1.4	3.6	1.6
	180-200	2.7	1.4	2.0	1.4	1.0	0.6
60	0- 20	46.2	50.3	50.3	47.6	48.6	2.0
	20- 40	14.7	13.4	12.7	14.7	13.0	1.0
	40- 60	4.7	6.0	6.0	7.4	6.0	1.1
	60- 80	4.0	3.4	4.7	4.7	4.2	0.6
	80-100	3.4	2.7	4.0	3.4	3.4	0.5
	100-120	3.4	2.7	2.0	2.0	2.5	0.7
	120-140	4.7	2.0	3.4	3.4	3.4	1.1
	140-160	2.7	2.7	4.0	1.3	2.7	1.1
	160-180	4.0	2.7	2.7	1.3	2.7	1.1
	180-200	2.7	2.7	3.4	2.0	2.7	0.6
63	0- 20	58.3	59.0	57.0	57.0	57.8	1.0
	20- 40	25.5	26.8	26.8	27.5	26.7	0.8
	40- 60	18.1	18.8	18.0	17.4	18.1	0.6
	60- 80	15.4	16.8	17.4	18.8	17.1	1.4
	80-100	11.4	10.7	8.7	10.0	10.2	1.2
	100-120	8.7	7.4	8.7	8.0	8.2	0.6
	120-140	6.7	5.4	7.4	7.4	6.7	0.9
	140-160	6.0	5.4	2.0	4.0	4.4	1.8
	160-180	4.7	3.4	4.0	4.0	4.0	0.5
	180-200	3.4	2.7	4.0	5.4	3.9	1.1
66	0- 20	69.7	70.4	70.4	71.7	70.6	0.8
	20- 40	36.2	43.6	44.9	50.3	43.8	5.8
	40- 60	33.5	31.5	33.5	34.8	33.3	1.4
	60- 80	23.5	21.4	22.8	22.1	22.5	0.9
	80-100	14.1	14.7	15.4	14.7	14.7	0.5
	100-120	10.7	12.1	10.7	8.7	10.6	1.4
	120-140	8.7	6.7	6.0	7.4	7.2	1.2
	140-160	4.7	6.0	6.4	5.0	5.5	0.8
	160-180	4.0	4.4	4.7	5.4	4.7	0.8
	180-200	5.4	4.7	4.3	4.7	4.8	0.4

comparable plateau is reached in Meckel's cartilage at a depth of 140-160μm below the articular surface, this level of cellularity deep in the tissue remaining relatively unchanged throughout metamorphic climax.

A similar variation is also described by Stockwell (1979) in mammalian cartilage where the tissue is not limited by a perichondrium, for example, beneath an articular surface. It therefore appears that the cellular architecture of Meckel's cartilage in Xenopus beneath the articular surfaces is quite similar to that of mammalian articular cartilages.

Although the high cell density of the surface zone is consistent with the proximity of the nutritional supply from the synovial fluid, no clearly defined function has been ascribed to these cells. Weiss et al (1968) suggested that they may have a fibroblastic role, helping in the maintenance of the surface zone of the matrix, while Stockwell and Meachim (1973) postulated that they may help to maintain a suitable metabolic environment for the deeper cells.

Despite the higher number of cells present in this superficial zone, it is more permeable than the deeper areas of the cartilage (Stockwell, 1979) and this may help to account for the dramatic increases in cell numbers present in these deeper zones between Stages 60 and 66NF. for which the deeper cells require an adequate supply of metabolites.

SECTION IV MATRIX PRODUCTION DURING METAMORPHOSIS

There is a considerable increase in the amount of matrix present in Meckel's cartilage during metamorphic climax. To investigate the time of commencement and rate of production of matrix, the changes in volume fraction of the cartilage occupied by matrix were assessed from Stage 57 to 66NF using a point-counting technique.

A METHOD

1 Preparation of material

This study was carried out using the same material as used in Section III.

2 Measuring technique

Ten sections from each of the four animals were selected randomly, and projected at an initial magnification of x 100 on to a screen at a distance of 210cm, using a Leitz Prado 250/50 microprojector. This resulted in a total magnification of x 850. (Prado, Instructions).

A 100-point square array grid (point separation 1cm) was superimposed on the image at two sites, chosen at random, this having been shown by Hilliard and Cahn (1961) to produce more accurate results than systematic sampling, and the number of points falling on the matrix recorded.

This figure was used to assess the volume fraction

occupied by matrix, using the methods described by Aherne and Dunnill (1982).

Although the matrix is not randomly distributed throughout the cartilage, Aherne and Dunnill (1982) state that volume fractions can be determined without modification of the basic techniques whether the structure in question is isotropic or anisotropic.

B RESULTS

The results are presented in Table IV_1 and Fig 48.

C DISCUSSION

Point counting to measure the area fractions of transected structures on microscopic sections is a basic morphometric technique, the most important use of which is the estimation of volume fractions, combining the point counting with the "Principle of Delesse". Delesse (1847) studying the make-up of composite rocks, showed mathematically that the area occupied by any one mineral on the surface of a section of rock is proportional to the volume of that mineral in the rock. As fractional areas can be readily determined, this principle has enabled volume fractions to be assessed more easily than by serial section reconstruction. If point counting is used as the technique for area estimation, certain criteria must be observed if accurate results are to be obtained.

TABLE IV_1

Stage	A	B	C	D	Mean	Standard Deviation
57	15	17	17	17	17	± 1.0
58	16	18	18	18	18	± 1.0
59	20	20	21	19	20	± 0.8
60	20	20	22	18	20	± 1.6
61	21	22	22	21	22	± 0.6
62	22	22	23	22	22	± 0.5
63	28	32	31	30	30	± 1.7
64	45	40	44	44	43	± 2.2
65	51	52	52	51	52	± 0.6
66	61	63	64	64	63	± 1.4

VOLUME FRACTION OF MATRIX (%)

1 Processing of tissue

Methods of tissue processing for histological examination result in some degree of distortion, and if absolute values for volume fractions are required, appropriate correction factors must be applied to any measured results. However, in this part of the study, only a comparative investigation was undertaken with no attempt being made to calculate absolute values. As the processing was identical for specimens at all Stages, no correction factor was applied, although it must be recognised that the tissue density at Stage 57 and Stage 66NF is different, and therefore some difference in the reaction and distortion might be expected, it has been assumed that these effects are much smaller than the changes being measured.

However, Weibel et al (1969) working with liver cells showed that considerable swelling of fixed cells occurred if dehydration was begun in concentrations of ethyl alcohol less than 70%. Since the cells in Meckel's cartilage are very different in size before and after metamorphosis, such swelling could affect the results obtained at Stage 57NF as compared with Stage 66NF, and particular care was therefore taken over this stage of processing.

2 Number of points counted

The number of points which require to be counted has been discussed by several authors. Chalkley (1943) in the

paper introducing the current methods of point counting, used a grid of only four points and from a statistical evaluation of his results claimed an accuracy of 5% with a total of 500 points counted. More recent work has however indicated that many more points must be counted if the results are to achieve this level of accuracy. Dunnill (1962) carried out numerous repeat counts and stated that 2000-3000 points must be used for a level of $\pm$ 5% accuracy. Hally (1964) worked out a mathematical formula which was based on the concept that the number of points to be counted depended on the volume fraction of the tissue component under investigation. This work was further developed by Anderson and Dunnill (1965) who, working on a comparative study of normal and emphysematous lung tissue, produced nomograms showing the relationship between the total number of points sampled, the estimated percentage volume fraction of tissue and the standard error of the estimate. As the appearance of alveolar sacs in lung tissue is not dissimilar to the net-like appearance of the matrix in Meckel's cartilage in Xenopus in the earlier Stages of metamorphosis, it was felt that this study was particularly pertinent to the present investigation. This method required an estimate of the volume fraction to be made before the number of points which must be used for a given accuracy can be determined. For this study therefore one animal at each Stage was used for a preliminary assessment. Ten sections were counted, using 100 points to

obtain an estimate of the matrix volume fraction. As the accuracy of the count is less the smaller the volume fraction, Stage 57NF was selected. At a volume of 20% Anderson and Dunnill (1965) showed that to obtain a standard error of not more than 5%, 2000 points must be counted. This number was therefore chosen for this study.

3 Geometry of the grid

Frolov and Maling (1969) discuss the mathematical principles underlying the efficiency of various geometrical arrangements of points on a counting grid. Three grid patterns, the triangular, the hexagonal and the square arrays are discussed. For a given point spacing the triangular grid is claimed to be the most efficient as it provides most points per unit area. However, the accuracy of the result is dependent on the total number of points counted, not the number per unit area. Therefore to obtain the same total number of points, a larger sample of a given tissue must be covered by the square array, and this was chosen for this study because, especially at the earlier Stages, the amount of matrix present is relatively small.

4 Point spacing

The spacing of the points on the grid must be of a suitable order of magnitude for the chosen method of counting (Aherne and Dunnill, 1982). It was decided that greater

accuracy could be attained by projecting the image than by counting directly with an eyepiece graticule, and a point spacing of 1cm was chosen as suitable for the scale of this projected image.

From the data presented in Table IV_1 and Fig 48, the volume fraction of the matrix remains unchanged during Phases I and II of metamorphic climax. It is only during Phase III, when the peak rate of cell division has passed, that any marked change occurs. However, when the matrix volume fraction begins to increase, it does so very rapidly, doubling the amount of matrix present in approximately 7 days (Stages 63-66NF).

It would appear that this increase in the matrix volume fraction is associated with the change in feeding habits commencing at Stage 64NF. Person and Philpott (1967) however describe a similar matrix increase during development of the gill cartilage of Limulus polyphemus, and explain this change as being associated with the increase in size of the animals. Meckel's cartilage in Xenopus, although changing shape, is not increasing in size during this phase (Shaw, 1982) and this explanation is therefore less likely.

SECTION V SITES OF MITOTIC ACTIVITY DURING THE DIVISION CLIMAX PHASE STAGES (60-62NF)

The number of cells in Meckel's cartilage increases dramatically during this phase, a period of some 48 hours. Therefore an attempt was made to localise the areas where activity was most marked. This was carried out in two ways.

A CONTROL SPECIMENS

1 Method

All material at Stages61 and 62NF prepared for Sections III and IV was re-examined. Every microscope section was searched at a magnification of x 400 for nuclei in metaphase or anaphase (Figs 49 a and b). Where such a mitotic figure was seen, its distance from the nearest part of the perichondrium was assessed by superimposing a grid of lettered squares on the field of view and noting the letter of the appropriate square, square A being placed with its outer edge along the perichondrium/cartilage interface. At this magnification, the sides of the squares were 20μm in length, and thus the dividing cells could be grouped into bands 20μm wide.

2 Results

The results are presented in Table V_1 A and B.

3 Discussion

The diameter of Meckel's cartilage varies from 160-200μm

TABLE V_1 A and B

A STAGE 61NF	A	B	C	D	TOTAL
Band A 0-20µm	9	7	6	10	31
Band B 20-40µm	3	4	2	3	12
Band C 40-60µm	1	-	2	1	4
Band D 60-80µm	-	-	1	-	1

B STAGE 62NF	A	B	C	D	TOTAL
Band A	14	12	10	12	48
Band B	5	3	2	4	14
Band C	1	-	1	1	3
Band D	-	-	-	1	1

MITOTIC FIGURES RECORDED AT STAGES 61 and 62NF

and it would appear that the main site of mitotic activity is subperichondrial but the numbers of mitotic figures observed are very low. Shaw (personal communication) stated that the principal times of cell division appeared to be around 03.00 hours and 15.00 hours. It was therefore decided to attempt to arrest the dividing cells using a stathmokinetic drug to obtain a more precise assessment of the division sites.

B EXPERIMENTAL SPECIMENS

Bretscher (1949), investigating the gross effects of colchicine applied topically at a concentration of 1:2000 to the developing rear limb bud of Xenopus, describes mitoses in the mesenchyme and epidermis, and describes the drug as causing morphologic changes by stopping cell division and causing cell death.

Créźé (1950) exposed Xenopus tadpoles aged one month (Stage 54-55NF) to colchicine at a concentration of 1mg/5L to examine its effect on the sequence and rate of metamorphic changes. He commented that Xenopus tadpoles were particularly sensitive to the drug, much higher concentrations producing no effect on the tadpoles of Rana, but did not state whether colchicine used in this concentration was effective as a stathmokinetic agent.

1 Method

Colchicine* was therefore chosen for this study, and a

*Sigma Chemical Co., London

total of 12 animals were used to find the optimum concentration for mitotic arrest. These animals were used in groups of 3, the 3 animals most closely conforming to NF Stage 61 at 12.00 hours being used.

The animals in each experimental group were transferred to individual beakers containing100ml of the appropriate concentration of drug, and kept at a constant 25°C for 24 hours, being illuminated for 8 hours (09.30-17.30 hours) by artificial light. They were then killed and prepared for examination with the light microscope, as described in Section I.5. Various strengths from 1mg/100ml to 1mg/5L were used and the most suitable strength was found to be 1mg/L. Above this level there was tissue damage as well as arrested mitotic activity (which was also difficult to recognise) while below, the tissue was indistinguishable from the untreated Stage 61NF material.

2 Results

Using colchicine at this concentration resulted in a quite large number of cells arrested during mitosis, although the individual cells are still found scattered throughout the peripheral zones of the cartilage. Cells exhibiting arrested mitotic figures were also observed in other tissues, especially in the epithelium. The mitotic figures are also more prominent following the use of the drug (Figs 50 a & b), and this group was therefore used for analysis. The results

TABLE V_2

ANIMAL	I	II	III	TOTAL
Band A	196	231	306	733
B	84	123	130	337
C	14	21	12	47
D	2	9	2	13

MITOTIC FIGURES RECORDED AFTER TREATMENT WITH 1mg/L COLCHICINE

are presented in Table V_2 and Fig 51.

These results confirm the observations on the untreated tadpoles, that the cell division is taking place near the periphery of the cartilage, and indicate that 1mg/L of colchicine is a suitable concentration for stathmokinesis in Xenopus tadpoles during metamorphic climax, without interfering dramatically with the development of the animals.

3 Discussion

Most work using stathmokinetic drugs has been concerned with a quantitative assessment of the mitotic index of various tissues, both normal and pathological (especially tumours). If the drug is being used in this way, rigid criteria must be applied if the results are to be really meaningful.

Tannock (1967) defined the properties that an ideal stathmokinetic agent should have, namely (1) at the dose level used and in the particular tissue under study, it should arrest all metaphases during the observation period; (2) these arrested metaphases should not degenerate into an unrecognisable state before the tissue is fixed and examined; and (3) the metaphase arresting ability of the agent should not have adverse effects on interphase cells. He concluded that none of the agents

tested was ideal, but that the vinca alkaloids were probably closer to it than colchicine and its derivatives. Subsequent work summarised by Aherne, Camplejohn and Wright (1977) has tended to support this view.

However, the present study is using the drug to "collect" mitotic figures, with the view to establishing the geographical locality of dividing cells rather than an assessment of the rate of cell division, and Tannock's views are less relevant than if the assessment of a mitotic index for the tissue were undertaken.

It is possible that the effect of the drug at the centre of the cartilage is limited by its ability to diffuse through the tissue, and therefore the number of mitoses observed in the deeper zones would be lower than the true figure. However, as the results following colchicine administration confirm the distribution of mitotic figures in the untreated tissue, it seems probable that the differences in numbers of dividing cells observed in the superficial and deeper zones of the cartilage represent a real phenomenon.

The results of this investigation could also be affected by the degeneration of arrested mitotic figures. Blocked metaphases have been shown to be functionally, morphologically and biochemically abnormal by Aherne and

Camplejohn (1972), and are therefore prone to break up and disappear. This degeneration will lead to an underestimate of the number of cells dividing after a period of about 6 hours (Aherne, Camplejohn and Wright, 1977).

It has therefore been assumed for the purposes of the present study that degeneration of arrested mitoses is occurring at a similar rate throughout the cartilage, and thus, although the total estimate of cells dividing will be lower than is in fact the case, the comparison of numbers of dividing cells at various depths within the cartilage will be unaffected.

A further disadvantage of colchicine and its derivatives quoted by Tannock (1967) is that anaphase and telophase figures are also observed at the optimal collecting dose, again affecting assessment of the mitotic index. This was not felt to be a problem in the present study, all dividing cells being relevant to the assessment.

Likewise the more recently introduced drug Nocodazole, although used successfully in Xenopus at the stage of somitogenesis (T G Elsdale, personal communication) was not thought to offer any advantage for the present study, as the conditions necessary for successful use of the drug are less suitable for free-swimming tadpoles.

Although this Section is concerned with the division climax phase, it was observed that prior to Stage 58-59NF the tadpoles would not tolerate a concentration of 1mg/L of colchicine, confirming Créze's statement (1950) that Xenopus is particularly sensitive to this drug, and also indicating that this sensitivity is not constant throughout the developmental period.

From the results presented above, it appears that cell division in Meckel's cartilage in Xenopus is similar to that observed in mammalian tissue, with interstitial growth taking place in the immediate subperichondrial zone.

Because of the very thin nature of the perichondrium and its tendency to separate from the cartilage during preparation of the tissue, it is difficult to assess what, if any, role appositional growth plays in the development of Meckel's cartilage. In areas where the perichondrium is fairly dense, and has remained attached to the cartilage, there is clearly a gradual transition from the very flattened appearance of the fibroblasts to the peripheral cartilage cells (Fig 49b), and this appearance is confirmed by electron microscopy (Fig 34). No recognisable mitotic figures were observed which were clearly in the perichondrium rather than in the cartilage, but it would appear that, as in mammalian cartilage, cells are being contributed to the periphery of the cartilage from the population in the perichondrium, which subsequently divide and function as chondroblasts.

An apparent increase in the number of cells dividing was observed at the articular ends of the cartilage and this correlates with the investigation of cell distribution below the articular surface described in Section III (Fig 47) where a dramatic increase in cell numbers in the corresponding zones is evident during metamorphic climax.

The lack of mitotic activity in the depth of the tissue correlates with the ultrastructural observations in Section III indicating that there is a significant population of apparently hypertrophic and degenerating cells even at the earlier Stages of metamorphosis.

During examination of the material for this Section, other tissues of the lower jaw, especially the epithelium were checked for the presence of arrested cell divisions. A significant number were observed in the epidermis and oral epithelium, and it therefore seems that colchicine at a concentration of 1mg/L is a useful stathmokinetic agent for other tissues, as well as cartilage, in Xenopus after metamorphosis has commenced (Fig 52).

SECTION VI THE EFFECT OF THYROID HORMONE ON THE METAMORPHIC CHANGES IN MECKEL'S CARTILAGE

Thyroid hormone radically affects the sequence of changes during metamorphosis in many amphibia, especially in the earlier stages. It was therefore decided to carry out a preliminary investigation into the effects of short-term dosage with the hormone on the architecture of Meckel's cartilage in Xenopus.

A METHOD

From the batch of tadpoles used for Sections V and VI, three tadpoles were selected which reached Stage 57NF on the morning of the same day. Each was transferred to an individual beaker containing 150ml of distilled water. To the water in two of these beakers 150mg of dry thyroid powder (Porcine, Iodine 0.2%*) was added, the animal in the third beaker being used as a control, Fox and Irving (1950 b) having shown this concentration (1mg/ml) of hormone to be suitable for inducing changes, and well tolerated by the tadpoles. Three animals at Stage 60NF were similarly treated.

The techniques described in Sections III and IV were used to compare the changes in cell population and amount of matrix in the control and experimental animals.

B RESULTS

1 Appearance of head and lower jaw

* Sigma Chemical Co., London

(a) Stage 57NF animals (Figs 53A and B)

The rapid shrinkage of the head described by Deanesly and Parkes (1945) is very obvious, as is the precocious development of the front limbs. The development of a prominent "snout" is also characteristic of animals following thyroid administration, due partly to the greater prominence of the eyes. The lower jaw shape has changed from a shallow curve characteristic of the early Stages of metamorphosis to a much deeper U-shape similar to that seen in the later Stages in untreated animals. One extra animal was used at this Stage to provide a whole mount of Meckel's cartilage, to illustrate this change (Fig 54)

(b) Stage 60NF animals (Figs 55A and B)

At the end of the experimental period, the control animal had developed to Stage 62NF, and no differences in appearance between it and the experimental animals was apparent.

2 Changes in cell population

The techniques for measuring and analysis of results described in Section III were used.

(a) Stage 57NF

The control animal was still at Stage 57NF after the experimental period, tadpoles remaining in this Stage for approximately 4 days (Nieuwkoop and Faber, 1956) and therefore the previously established mean nuclear number

for Stage 57NF is included along with the present results in Table VI_{1A}. The appearance of the cartilage in the experimental animals was markedly different from that of the control, the lacunae appearing smaller and the amount of matrix present appearing much greater, and exhibiting a more intense staining reaction (Fig 56). The nuclear count for the control animal is within one standard deviation of the Stage 57NF mean, while the experimental mean has risen to a level previously reached at Stage 61NF following development for 7 to 8 days. Although this preliminary study has only used a very small experimental group, this difference is statistically significant ($p < 0.001$). However further work would be required to demonstrate more fully the biological changes which are occurring.

(b) Stages 60/61NF

The control animal, on re-staging, was at Stage 61NF and therefore the mean nuclear number for Stage 61 has been included in Table VI_{1B} for comparison. The histological appearance of the control and experimental animals was closely similar, any difference in the amounts of matrix present, size of lacunae and cell density appeared indistinguishable between the three animals (Fig 57).

3 Matrix production

The techniques for measuring and analysis of results described in Section IV were used.

TABLE VI_1

Control	Stage 57 mean	Experimental I	Experimental II	Experimental mean
23,892	24,536	32,538	34,642	33,570

A. 48 HOUR THYROID EXPERIMENT-STAGE 57
CORRECTED NUCLEAR NUMBERS

Control	Stage 61 mean	Experimental I	Experimental II	Experimental mean
38,024	37,052	39,928	37,188	38,558

B. 48 HOUR THYROID EXPERIMENT - STAGE 60/61
CORRECTED NUCLEAR NUMBERS

(a) Stage 57NF

The volume fraction of the cartilage occupied by matrix in the control and experimental animals, together with that previously assessed for Stage 57NF is presented in Table VI_{2A}, showing a very dramatic increase in the amount of matrix present, corresponding to the observed change in lower jaw shape.

(b) Stages 60/61NF

The amount of matrix present has not been affected by the exposure to the hormone, (Table VI_{2B}).

C DISCUSSION

Gudernatsch (1914) first showed that thyroid preparations could induce premature metamorphosis in some amphibians, and Allen (1916) demonstrated that thyroidectomy, if performed early in the life of the tadpole, prevented it. Following these observations, many workers have studied the effects of thyroidectomy and excess thyroid hormone on a range of amphibian species. Lenhart (1915) suggested that the induced metamorphic changes could be used for a qualitative assay of the activity of various thyroid compounds, and this work was continued by Gaddum, (1927) and Deanesly, Emmett and Parkes (1945) using tadpoles of Rana temporaria. However, the short breeding season in this species made it difficult to carry out detailed examination, and Deanesly and Parkes (1945) pioneered the

TABLE VI_2

Control	Stage 57 mean	Experimental I	Experimental II	Experimental mean
16	17	45	49	47

A. 48 HOUR THYROID EXPERIMENT-STAGE 57
VOLUME FRACTION OF MATRIX (%)

Control	Stage 61	Experimental I	Experimental II	Experimental mean
21	22	22	23	23

B. 48 HOUR THYROID EXPERIMENT-STAGE 60/61
VOLUME FRACTION OF MATRIX

use of Xenopus for such studies, noting that the sequence of changes during metamorphosis was altered, especially obvious being the very rapid reduction in head size early in metamorphic climax.

Hoskins (1922) transplanted the thyroid gland into the tails of tadpoles prior to metamorphic climax. As metamorphosis commenced, the tails, including the transplanted gland, were removed and the tadpoles were found to complete metamorphosis normally, suggesting that the tissues are prepared for metamorphosis by the action of thyroid hormone, but that it is not necessary for the process itself. All the experimental animals were examined histologically for evidence of regeneration of thyroid tissue in the normal site and those showing such evidence were eliminated from the study. However it is also possible to explain this result if regeneration of thyroid tissue occurs at other sites, or if the hormone can be stored in the tissues.

Etkin (1935) showed that the thyroid gland becomes increasingly active during early metamorphosis, and suggests that different organs have different thresholds of response to the action of the hormone. Working with thyroidectomised Rana cantabrigensis tadpoles (Op.cit.), he concludes that the thyroid functions during the premetamorphic period to prepare the tissues for metamorphic climax, but that continuing concentrations of hormone are required during this time

if the processes are to continue normally.

Allen (1932) deduced from his studies of Bufo tadpoles that different organs are susceptible to thyroid hormone only at specific stages in their development, and thus not only concentration but also time of administration is important.

In a review paper (Allen, 1938) he concludes that there is clear evidence that thyroid activity is maximal near the beginning of active metamorphosis.

Most workers have been concerned with the gross changes produced by interfering with normal thyroid function. The effects on the histology of the gland itself have been described by Lynn and Peadon (1955) and that on parts of the central nervous system in tissue culture by Schaffer (1963). It is therefore interesting to attempt to relate the changes produced at a cellular level in Meckel's cartilage with those occurring in other parts of the animal.

The dramatic stimulus to cell division and matrix synthesis provided by the hormone at Stage 57NF, associated with a much earlier change in shape of the cartilage agrees with Etkins' conclusion (1935) that higher concentrations of thyroid hormone speed up the metamorphic process.

However he also states that the sequence of metamorphic events is independent of the concentration used (in Rana cantabrigensis). However, Deanesly and Parkes (1945) and the results of the present study suggest that in Xenopus this is not the case, the process being not only accelerated, but the sequence of events changed, in that normally the front limbs erupt before the head begins to shrink, but this order is reversed in animals exposed to excess thyroid hormone.

The findings of this preliminary investigation are also in agreement with the concept that structures in the tadpole have a specific stage in development when they are affected by thyroid hormones. In the case of Meckel's cartilage this would appear to occur early in metamorphic climax (or shortly before climax commences), as Jurand (1959) has demonstrated that thyroid hormone plays no morphogenetic role until Stage 52-53NF has been reached. This susceptibility of the tissue is apparently lost by Stage 60NF.

This concept of a "time of action" of the hormones may in fact merely be an expression of the "threshold of action" as the particular time when the tissue responds may be the only time during development when the thyroid hormones are at the correct level or the receptors in the tissue functional and too high a hormone level could be as ineffective as too low.

The response of Meckel's cartilage prior to Stage 60NF agrees with the concept that the main thyroid activity occurs early in metamorphosis, correlating with Etkins' (1935) findings that the gland exhibits increased activity at this time.

Lenhart (1915) stated that many of his experimental animals developed a condition of generalised body oedema if exposed to too high a concentration of iodine, a problem not encountered in this study. Iodine concentration may be the explanation of the disease described by Müller (1976) encountered in Xenopus tadpoles, where the lymph sacs become engorged, the body becomes bloated and swimming unco-ordinated, leading to death within 2-3 days.

This preliminary work has also shown Meckel's cartilage during early metamorphosis to be highly sensitive to the action of thyroid hormones. The changes in this cartilage may make it a useful tissue for more detailed investigation of the "threshold" and "time of action" theories.

GENERAL DISCUSSION

1 Staging

The changes occurring leading up to and during metamorphosis in many species of amphibia have been summarised in the form of Normal Tables (New, 1966). Tables pertaining specifically to Xenopus have been published by Gasche (1944), Weisz (1945 c) and Bretscher (1949),none of which provide very detailed accounts of the process. However, the tables produced by Nieuwkoop and Faber (1956) dealing with Xenopus laevis (Daudin) give a much more satisfactory description than any of the previous ones. Nieuwkoop and Faber provide, in addition to a comprehensive survey of the changes occurring in all the major body systems, a detailed table based on external and internal criteria dividing the development into 66 precisely defined Stages.

Metamorphic climax occupies Stages 58-66 using the Table of Nieuwkoop and Faber. During this phase the tadpoles are passing through each Stage rapidly (Table 1) and very careful judgement is required if reasonable accuracy in staging is to be achieved. The features used for staging are constantly changing and this, when associated with the normal variation in rate of development and appearance in different individuals, means that it is rare to find an animal in which all the features correspond exactly to the defined criteria for a particular Stage. Particular care has therefore been taken to ensure that the animals selected

conform as closely as possible to the desired developmental Stage. All external features were used, internal features being inaccessible for staging without microdissection or histological examination.

The Nieuwkoop and Faber Table also quotes a range of lengths for each Stage. The lengths of the tadpoles used in this study were found as metamorphic climax was approached to be considerably shorter than those stated although corresponding accurately to the other criteria. This difference, which frequently was considerable as climax was reached, probably arises because Nieuwkoop and Faber based their Table on animals collected from a single natural location, whereas the breeding animals used for producing the tadpoles used in the present study are derived from a strain bred in laboratory conditions for many generations. The lengths quoted by Jayatilaka (1978) in animals approaching metamorphic climax correspond to the measurements of similar animals used in this study, rather than those quoted by Nieuwkoop and Faber (1956). Tadpole length has therefore been ignored for assessing development up to Stage 57NF, the length not being a staging criterion once climax has commenced.

The rate of development of the tadpoles was found to be closely similar to that described by Nieuwkoop and Faber (1956).

2 Electron Microscopy

The technique devloped for fixing this immature amphibian cartilage, and described in Section I is important, as this tissue appears to be particularly prone to osmotic destruction with most fixatives tried.

Osmium tetroxide, at a strength of 1%, was found to be the most satisfactory agent, and was initially used dissolved in frog Ringer solution. This resulted in the nucleus and nucleoli being fixed, but the cytoplasm was represented as a loose fibrillar material with no evidence of endoplasmic reticulum, Golgi apparatus or ribosomes. The nuclear envelope revealed the perinuclear cisternae to be dilated, the outer membrane to be studded with ribosomes and the presence of prominent nuclear pores. Fawcett (1981) and Carr and Toner (1982) indicate that in a number of cells, early biosynthesis of new products occurs in this area. It seems improbable that the great increase in matrix production during metamorphosis is mainly due to synthesis in this area of the cell alone as such activity in the cisternae is normally associated with a well-developed granular endoplasmic reticulum, with direct communication between their lumina.

Goin and Goin (1971) describe amphibian tadpoles developing in fresh water as constantly taking in water through the skin and excreting large amounts of dilute urine to remove

the excess, and Sedra (1949) describes Rana tadpoles losing 60% of their body weight at metamorphosis mainly because of water loss. As the living animals contain so much water, it was decided to use the technique of Sprinz and Stockwell (1976), dissolving the osmium tetroxide in water, with no addition of ions or buffers and this method of fixation finally produced acceptable results.

These problems of fixation indicate that immature cartilage in Xenopus is a particularly sensitive tissue to the osmotic and chemical actions of fixatives. However, adult cartilage in other amphibians (Dickson 1978, 1982) as well as larval thyroid tissue (Jayatilaka 1978) can be satisfactorily fixed with a number of standard techniques. It is unclear why immature amphibian cartilage is so susceptible to processing damage. However, it is speculated that it may be due in part to the very small amount of matrix present and the delicate water balance maintained in tadpoles.

3 Phases of development

Combining the results obtained in Sections III and IV it is possible to build up an overall picture of the architectural changes occurring in Meckel's cartilage (Fig 58). The three phases into which metamorphic climax could be divided, which were presented in Section III, can now be described more fully.

A. Phase I comprises NF Stages 57 to 60, during which there is a slow increase in cell numbers but very little change in the matrix volume fraction - the "Lag" phase (Day 41-46).

B. Phase II, during which there is a rapid increase in cell numbers still with little change in the amount of matrix - the "Division" phase (Day 46-49, NF60-62).

C. Phase III, during which there is a very rapid increase in the amount of matrix, but a levelling off in the rate of cell division - the "Synthesis" phase. (Day 49-58, NF62-66).

4 Lag phase

At the outset of this phase the cartilage is a shallowly curved bar, convex anteriorly, with little matrix present. Its articulation with the chondrocranium is placed anteriorly, and in the living animal its structural rigidiity is low. However, at this time the tadpoles are filter feeders, and the lower jaw is acting simply as a flap valve, controlling the entry of water and suspended particles into the anterior part of the alimentary canal.

Grove and Newell (1966) describe the developing notochord being composed of vacuolated cells, surrounded by a fibrous sheath. The turgor pressure of the cells, acting

against this sheath, results in the notochord forming a stiff, but flexible rod. It seems probable that such rigidity as Meckel's cartilage possesses at this stage of development arises in a similar way, the perichondrium having an analogous role to the notochordal sheath.

During the phase there is little change in the internal organisation of the cartilage although there is some reduction in width and increase in antero-posterior length.

The cellular organisation described at the ultrastructural level appears to be related to the approaching division phase, with the "reserve" zone present on the periphery of the bulk of the cartilage, and the presence of "proliferative" zone cells in specific sites. It is interesting that Meckel's cartilage shows analogous features to the growth cartilage in the frog described by Dickson (1982) in its cellular organisation, as in mammals it shows no such similarity.

The presence of apparently hydropic and degenerating cells, and cellular debris in some zones within the cartilage is particularly interesting at this early stage in the development of the tissue, although the possibility of poor penetration of fixative into some areas could be responsible for this appearance.

Glucksmann (1951) states that cell death occurs in many

organs and tissues including cartilage from their earliest developmental stages, and probably plays an important part in their morphogenesis. He describes changes in the shape of organs being brought about by integration of cell division, cell death and cell movement. He also indicates that degenerating cells would be expected in amphibia, associated with removal of, or changes in shape in, larval organs during metamorphosis, and Bowen and Lockshin (1981) state that virtually every tissue in the amphibian body undergoes extensive remodelling during this time.

This type of cell death, where selected individual cells are deleted to the advantage of the rest of the organism, dubbed "apoptosis" by Kerr, Wyllie and Currie (1972) is distinct from necrosis, the other major type of cell death, and is increasingly coming to be regarded as an important process in embryogenesis and metamorphosis in amphibians and insects (Bowen and Lockshin, 1981).

The distinctive features of apoptosis in mammalian tissues are summarised by Bowen and Lockshin (1981) as loss of cell junctions, condensation of cytoplasm, clumping of nuclear chromatin to form several discrete masses and ultimate break up of the cell to form a number of membrane-bound apoptotic bodies which are either extruded into an adjacent lumen or undergo phagocytosis. It is possible that the granular debris observed during this phase

(Figs 31 and 32) may represent such structures.

Isolated single cells within a tissue rather than fields of contiguous cells tend to be involved.

Although loss of cell junctions and extrusion or phagocytosis of apoptotic bodies seem unlikely in cartilage, no cells exhibiting other features of apoptosis were observed in Meckel's cartilage. However Bowen and Lockshin (1981) state that in lower animals quite different morphological forms of apoptosis may exist and certainly it is isolated cells within Meckel's cartilage in Xenopus which are affected.

It is therefore possible that the evidence of cellular degeneration and cell debris observed during this phase represents a form of apoptosis; it would certainly seem to be covered by the expression used by Duvall and Wyllie (1983) to describe apoptosis - "physiological cell death".

Meckel's cartilage is undergoing some change in shape during this phase (Shaw, 1982) and the presence of such apoptotic cells is in accord with Glucksmann's hypothesis (1951). Although there is considerable evidence that the cartilage cell is constantly altering its outline by extending and retracting its processes (Chesterman and Smith, 1968) there is no evidence that the cells move bodily through the matrix, therefore the changing shape of

Meckel's cartilage during this lag phase when the amount of matrix present remains virtually unchanged seems to be mainly due to a balance between the cell division and death occurring within it.

5 Division phase

During this quite short period in the development of the cartilage (Day 46 to Day 49 post fertilisation, Stages NF 60-62) there are, in addition to the very rapid increase in cell numbers, dramatic changes taking place in the whole tadpole. The greatest increase in lower jaw length and decrease in width takes place (Shaw, 1982) at the same time as a considerable relative backward migration of the jaw articulation on the base of the chondrocranium is occurring (Sedra and Michael, 1957). These changes result in a considerable increase in the potential gape of the jaws. There appears at this stage to be little change in the amount of matrix present, and it seems likely, as hydropic and degenerating cells are still present, that again the changes in shape are mainly due to cell division and cell death occurring.

The main locus of dividing cells seems to be on the periphery of the cartilage, correlating with the ultrastructural observations of zones of proliferating cells lying subperichondrally in many situations. The degenerating cells appear to be mainly in the deeper zones of the

cartilage. Such degenerating cells are frequently located alongside apparently normal ones, however it is possible that this appearance may be due to local differences in the rate of penetration of the fixative, or differences in the metabolic state of the cells, resulting in some undergoing more rapid degenerative changes.

The changes occuring in the lower jaw during this phase are accompanied by dramatic changes in the morphology and structure of the alimentary canal, which is undergoing complete reorganisation, including considerable shortening of the intestine, and increase in stomach size (Nieuwkoop and Faber, 1956). This reorganisation is probably associated with the change of diet occurring during metamorphosis.

6 Synthesis phase

This phase is characterised by a very rapid increase in the matrix volume fraction of the cartilage, which is accompanied by further changes in shape, particularly a continuing antero-posterior lengthening of the jaw. During this time, the cellularity of the tissue is continuing to increase very slowly, the rate decreasing as the end of the phase is approached. Some degenerating cells are still present in the deepest zones although there is now no evidence of a subperichondral zone of

actively proliferating cells. This zone now appears to consist of considerably less densely packed cells than in the Lag and Division phases except at the site of the articulation with the chondrocranium. As the change in cell numbers present is relatively small, it seems probable that the alteration in shape during this phase is mainly due to the rapid synthesis of matrix. The lysosomes which are by this stage a feature of the cells suggest that matrix turnover is also an important factor.

A sharp increase in cellularity as the joint surface is approached is a feature of articular cartilage in mammals (Stockwell, 1971) and has been related both to the available nutritional supply and the mechanical properties of the tissue. In Meckel's cartilage in Xenopus at the end of the Synthesis phase, such an increase is very marked (Fig 18), a feature also seen in the opposing joint surface of the quadrate. This cellularity is not so striking a feature of the previous phases. As the dimensions of the articular surfaces do not appear to alter dramatically during metamorphosis, it seems unlikely that the increase in cellularity is related to any appreciable extent to nutritional factors, except in so far as the proximity of nutrients makes it possible for cell density to be increased here rather than elsewhere.

The change in the shape of the joint during metamorphosis

is quite striking. At Stage 57NF (Fig 13) the articular surface of the quadrate is flat or convex, while that of Meckel's cartilage tends to be concave. By Stage 66NF (Fig 18) the joint is a much more regular shape, the quadrate surface being concave, with the convex end of Meckel's cartilage corresponding closely in curvature.

The precise configuration of the joint surfaces at Stage 57NF appears to be quite variable, and may reflect the very small amount of matrix present at this Stage.

Feeding, which is considerably reduced during the Lag and Division phases recommences as the end of this phase is approached. However, now the adult predatory feeding pattern has replaced the filter feeding of the tadpole.

7 Ossification

From the commencement of the Division phase, membrane bones (dentale and goniale) are starting to ossify around Meckel's cartilage, although no areas of endochondral ossification have been observed within the cartilage up to the end of metamorphosis (NF Stage 66). The development of these bones has been described by Paterson (1939), Nieuwkoop and Faber (1956), Sedra and Michael (1957) and Shaw (1982). None of these authors has attempted to measure the amount of bone present, but all state that by Stage 66NF (the end of the Synthesis phase), the cartilage is surrounded

by the developing bone, except at the articular surface and a small area in the midline. In the animals used for this study, the cartilage is by no means completely surrounded by bone, even at the end of the Synthesis phase. The greatest amount of bone present is on the postero-medial aspect of the cartilage, about 40-50μm in thickness.

Although to produce the best histological results at the end of metamorphosis, a short period of decalcification would have been advantageous, this has not been done with the material used for quantification in this study, to ensure comparability with undecalcified tissue from the earlier stages. The limited decalcifying action of Bouin's fixative ensured adequate softening of the hard tissues and facilitated the preparation of serial sections with minimal damage.

It would therefore appear that the rigidity of the lower jaw is being increased during metamorphic climax by both an increase in volume fraction of cartilage matrix and the accretion of a partial collar of membrane bone around Meckel's cartilage.

This pattern of ossification is similar to the type of ossification seen in the early development of the mammalian long bone where a collar of membrane bone is laid down around the intact cartilage model, prior to

endochondral ossification commencing, as summarised by Stockwell (1979). It is possible that Meckel's cartilage in Xenopus may be a useful model for studying the changes taking place in this very early ossification process.

8 Feeding

A change in the feeding habit during metamorphosis is common to many species of amphibia, but is particularly marked in Xenopus and other species with filter-feeding tadpoles. In such species the lower jaw requires very little rigidity to be supportive in feeding. However in tadpoles which feed by scraping off the surface layer of the available food, as described for example in Bufo regularis by Sedra (1949), considerable structural strength is required. In these animals,from the early tadpole stages, the lower jaw requires a degree of rigidity and both jaws are also covered by horny plates along their margins to assist the scraping mechanism (Goin and Goin, 1971). The absence of separate suprarostral cartilages in Xenopus tadpoles is also attributed by de Beer (1937) to the pattern of feeding.

By Stage 64NF, many tadpoles start to feed aggressively, both dead and sickly animals being attacked. The prey at this stage is dealt with entirely by the jaws, the hind limbs not yet appearing to play any part in tearing up the food. This suggests that the changes in shape

and structure of Meckel's cartilage are concerned with preparing the lower jaw and its articulation with the quadrate for this new and more robust function, requiring greater rigidity and strength of the lower jaw.

On the upper jaw, by Stage 64NF most teeth have perforated the oral epithelium (Nieuwkoop and Faber, 1956). These teeth only protrude a few microns through the surface and do not occlude with the lower jaw, which closes into a groove situated just posterior to them (Shaw, 1982). It therefore seems that the gripping of prey depends on a rigid lower jaw closing into this groove, though not upon the presence of the teeth. This gripping action is important in feeding, as quite large items of prey are tackled, the food being captured in the jaws and then fed into the mouth in stages by the "hands" before being swallowed whole, a mechanism which requires a firm lower jaw. The increased potential gape of the jaw is also important in dealing with large items of prey.

The change in the articulation between Meckel's cartilage and the quadrate area from a loose, irregular joint at Stage 57NF to a more regular, close-fitting arrangement at Stage 66NF is probably also associated with this change in feeding habit.

Although lying so close to Meckel's cartilage in the

lower jaw, the ceratohyal cartilages do not undergo similar structural changes during metamorphosis, which suggests that they are not required for feeding or support at this stage.

9 Quantification of results

The quantification of the results in this study, presented in Sections III and IV, has been used to describe the events taking place during metamorphosis in Meckel's cartilage more precisely than is possible by a qualitative description of the changing appearance.

The technique described for counting nuclei was found to result in more accurate repeat counts than methods using an eyepiece graticule.

The discrepancy between the actual section thickness and the microtome micrometer reading is particularly interesting, being twice previously quoted figures (Marengo, 1944). The method of measuring section thickness quoted by Konigsmark (1970) using oil immersion was not found in this study to result in a more accurate measurement.

The question of section thickness is particularly important if an absolute value for cell number is being sought. However, the quantification of this and matrix volume fraction in this study was only used as a means of

comparison of different Stages, not to determine absolute values.

This quantified approach to the events occurring during natural metamorphosis provides a basis for future experimental work.

10 Drug administration

Suitable dose levels of colchicine and thyroid powder have been established for short-term experiments on Xenopus tadpoles.

(a) Colchicine

The particular sensitivity of Xenopus to this drug is curious, as Rostand (1950) working on Rana tadpoles, and Créze (1950) using Bufo, found doses of the drug, lethal to Xenopus tadpoles, had no apparent effect on the development. Créze (1950) describes a concentration of 1mg/5L added to the aquarium as lethal to the majority of his experimental group, the tadpoles developing an abnormal curvature at the base of the tail, leading to an inability to swim, and subsequently death. The animals which survived to complete the development of the hind limbs exhibited consistently an absence of the two medial digits on both limbs, the other digits, apart from the interdigital membrane developing normally.

It would seem therefore that colchicine has an effect on

the development of the skeletal system in Xenopus, accounting for the abnormal effects on the tail, and the agenesis of the two medial digits. Crezé (1950) administered the drug at approximately NF Stage 53, by which time the other digits have already begun development and appear to be unaffected, indicating that the drug is effective at a fairly precise stage in the development of the tissue. It is unclear why this effect is greater in Xenopus than other anuran species.

The dose level of 1mg/L required for stathmokinesis, although higher than that used by Crezé (1950) appears to be well tolerated by the animals for the 24 hour period, and results in a number of arrested mitotic figures. As these are observed in tissues other than the cartilage this dose level may be of more general application in studying the cellular dynamics in Xenopus tadpoles. Further analysis of these in Meckel's cartilage, sectioned in different planes are required to confirm the conclusions reached in Section V about the geographical location of the dividing cells.

(b) Thyroid powder

Comparison of the cell numbers in the control animals, and those previously established in Section III, indicates that those in the thyroid control tadpoles are within one standard deviation of the mean value, confirming that the cell numbers measured during normal metamorphosis may form

a useful basis for further study.

The dramatic effects of thyroid administration early in metamorphic climax, with early shrinkage of the head and premature Division and Synthesis phases in Meckel's cartilage, compared with its apparent lack of effect a short time later (5-6 days) is interesting. It may be possible to use the changes produced in Meckel's cartilage to determine the validity of the ideas of "time of action" and "threshold action" or to demonstrate whether they represent two facets of the same concept.

The long term effect of the dose of 1mg/ml thyroid powder on the subsequent development and growth of the animals has not been established.

11 Invertebrate cartilage

It is now generally accepted that true cartilage occurs in invertebrates, and the literature is reviewed by Person (1983). Cartilage occurs in many groups of invertebrates, including Coelenterates, Annelids, Molluscs and Arthropods. The types of cartilage found in the latter two groups range from types resembling vertebrate hyaline cartilage with relatively small numbers of cells embedded in an abundant matrix to extremely cellular cartilages with virtually no matrix (Person, 1983). The odontophore cartilage of Busyon canaliculatum (Gastropoda) described by Person and

Philpott (1967) is of this latter type, and is strikingly similar to Meckel's cartilage in Xenopus. The branchial cartilages of Limulus polyphemus (Arthropoda) are in the young animal also very similar to Xenopus, consisting of large numbers of cells separated by thin rims of matrix. As the animal grows, the cells increase in size, as does the thickness of the matrix rims, until, in the adult the tissue resembles hyaline cartilage (Person and Philpott, 1969). This change during growth seems to be very similar to the changes occurring in Meckel's cartilage in Xenopus during metamorphic climax.

12 Meckel's cartilage and plant tissues

Virchow (1858) stated that many types of cartilage bear a close resemblance to plant tissues, a view also expressed by Siegel (quoted by Person, 1983), who pointed out that cartilage and the skeletal plant tissues have developed in response to a common evolutionary need to resist and work against gravity. The walls of these plant cells, thickened by cellulose or lignin, correspond to the matrix of cartilage.

Plant parenchyma is a tissue consisting of thin-walled normally turgid cells which maintain the firmness of structures e.g. leaves (Roberts, 1971) by a mechanism similar to that proposed for the premetamorphic Meckel's cartilage in Xenopus where the amount of matrix present is

low. Collenchyma, where the cell has an unevenly thickened wall of cellulose, and sclerenchyma where the wall is uniformly thickened by lignin (Roberts, 1971) derive their skeletal properties from these walls, a rather similar situation to that in Meckel's cartilage after metamorphosis, where considerably more matrix is present.

In conclusion, this thesis describes the changes taking place in the morphology of Meckel's cartilage during normal metamorphosis in Xenopus laevis.

A technique for satisfactorily fixing the immature cartilage for electron microscopy has been developed, and a suitable dose level of colchicine for stathmokinesis during metamorphic climax has been described.

A preliminary study of the effect of thyroid administration during metamorphic climax has been carried out.

An attempt has been made to quantify these changes and correlate them with the different functions of the lower jaw before, during and after metamorphosis.

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APPENDIX 1

DETAILS OF TECHNIQUES USED

A Preparation of whole mounts

1 Lower jaws were removed and fixed in 100ml of 10% formalin for 24 hours.

2 The specimens were washed in 3 changes of 100ml of distilled water for 24 hours. Skinning is not necessary as there is very little pigment present in the skin of the lower jaw.

3 The specimens were stained in 100ml of a solution of 10mg alcian blue, 20ml of glacial acetic acid and 80ml of 95% ethyl alcohol for 24 hours.

4 The jaws were transferred through 100ml of 95%, 70% and 10% ethyl alcohol, 1 hour in each.

5 The jaws were transferred to 100ml of distilled water for 3 hours, changed 3 times.

6 The tissue was cleared in 100ml of an enzyme solution of 1g of beef trypsin (0.5 Anson units/g) 30ml aqueous sodium borate and 70ml distilled water for 48 hours.

7 The specimens were macerated in 20 ml of 0.5% aqueous potassium hydroxide, to which 5 drops of alizarin red S had been added, for 24 hours.

8 The jaws were washed in 100ml of distilled water for 3 hours, changed 3 times.

9 The specimens were transferred through 100ml of 25%, 50% and 75% glycerin and water mixtures, 24 hours in each.

10 The jaws were stored in 100% glycerin.

B Preparation for light microscopy

1 Lower jaws were removed and fixed in 100ml of Bouin's solution for 24 hours.

2 The specimens were washed for 1 hour in 3 changes of 100ml of distilled water.

3 The specimens were transferred through 100ml of 70% and 96% ethyl alcohol, 24 hours in each.

4 The jaws were transferred to 100ml of 100% ethyl alcohol for 24 hours.

5 Clearing the specimens was begun by placing them in 100ml of a solution of equal parts of methyl salicylate and 100% ethyl alcohol for 1 hour.

6 Clearing was completed by placing the jaws in 100ml of 100% methyl salicylate for 24 hours.

7 The specimens were placed in 100ml of methyl salicylate to which 1g of celloidin had been added.

8 The specimens were then transferred through 3 half hour changes of 100ml of CNP 30.

9 The jaws were then transferred through 3 half hour changes of paraffin wax in a vacuum embedder.

10 The specimens were individually blocked in paraffin wax, the blocks being cooled rapidly under running water, when the surface layer of wax had solidified.

11 The blocks were trimmed and sectioned at 10um on a Leitz rotary microtome. The sections were stained with Heidenhain's Iron Haematoxylin as described by Cullen (1979).

C Preparation for electron microscopy

1 The lower jaw was removed and cut in half. Each part was then immersed in 50ml of 1% osmium tetroxide dissolved in distilled water, for 24 hours.

2 The specimens were then placed in 50ml of 10% ethyl alcohol for 1 hour.

3 The specimens were transferred through 3 changes of 50ml of 100% ethyl alcohol for half an hour in each change.

4 The specimens were then placed in 50ml of epoxy propane for half an hour.

5 The tissue was placed in 50ml of equal parts of epoxy propane and araldite for 12 hours.

6 The tissue was placed in 50ml of araldite for 12 hours at room temperature.

7 The specimens were transferred to 50ml fresh araldite which was polymerised for 48 hours at 60°C.

8 lum sections were cut, stained with toluidine blue and examined, for orientation purposes.

9 Thin sections were cut at 80um on a Reichert OMU ultramicrotome using a glass knife, and the sections collected on copper grids.

Note: All material prepared for examination in the electron microscope was dehydrated and embedded using the same technique, only the fixatives (Stage 1) being changed. (Section I, 6).

D Staining procedure for electron microscopy

Stain solutions

1 Lead citrate solution (0.2%)

Lead citrate 0.2g

0.1N NaOH 100ml

To make the solution at 0.2%, 0.2g lead citrate is added to 100ml 0.1N NaOH.

2 Uranyl acetate solution

50% ethanol.....5ml

Uranyl acetate ...enough to saturate the solution (approx 1g)

To make the solution, sufficient uranyl acetate (1g) was added to saturate the solution of 50% ethanol. The solution was left overnight in the agitator and filtered before use.

Staining procedure

1 Grids were floated on to the lead citrate solution, sections 'face down' for two minutes.

2 Grids, held in forceps, were washed by gently moving them to and fro under the surface of distilled water for 10 seconds.

3 Grids were immersed in the solution of uranyl acetate for 10-15 minutes.

4 Grids, held in forceps, were moved under a solution of 50% ethanol for 20 seconds.

5 Sections were allowed to dry in air on filter paper before viewing.

APPENDIX II PUBLISHED WORK

Meckel's cartilage in Xenopus laevis during metamorphosis. D. A. R. Thomson, Anatomy Department, University of Edinburgh.

During metamorphosis, as well as a change in shape, Meckel's cartilage undergoes a significant change in structure. Prior to metamorphosis (up to Stage 57 Nieukoop and Faber) the tissue consists of small amounts of intercellular matrix surrounding large lacunae containing cells with prominent nuclei and mitochondria, and the remainder of the cell filled with a loosely packed fibrillar material. After metamorphosis (Stage 66) the amount of matrix has considerably increased, and the lacunae have decreased in size by about 75%. This is accompanied by an increase in cell number in excess of 50%.

The cells now appear to be of two types. Those near the periphery of the tissue have large nuclei and uniformly distributed dense cytoplasm containing prominent vesicles, mitochondria and Golgi apparatus. The more deeply placed cells have cytoplasm which is less evenly distributed and a relatively smaller nucleus. The intercellular matrix now clearly shows randomly oriented collagen fibres, with large numbers of small granules. The matrix at this stage comprises a significant volume within the tissue. These changes take place within a period of less than 20 days in animals maintained at 22-24°C.

It is probable that these changes can be associated with the alteration in feeding habits from the tadpole to the adult frog.

Abstract from I.A.D.R. British Division

April 1983

J Dent. Res. 62 4 432

Fig 1

XENOPUS LAEVIS (DAUD).

Adult female, dorsal aspect

Snout-vent length 107mm.

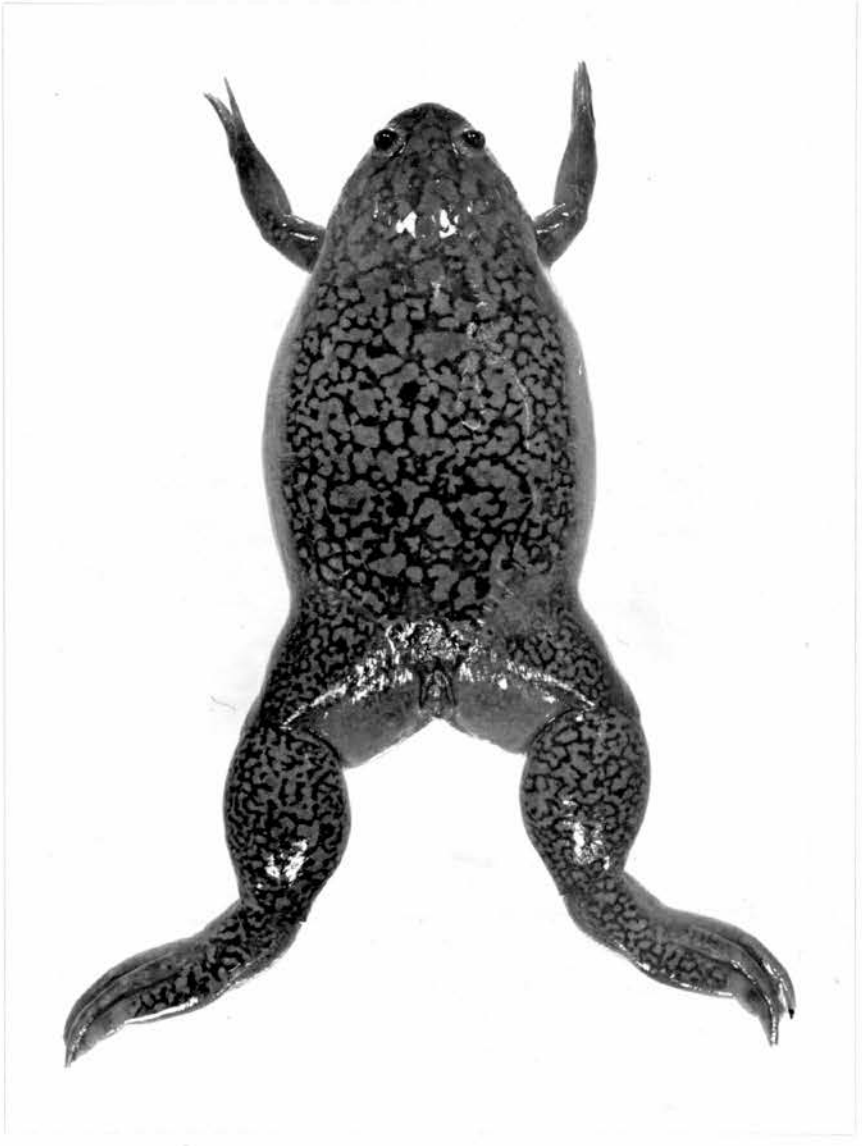
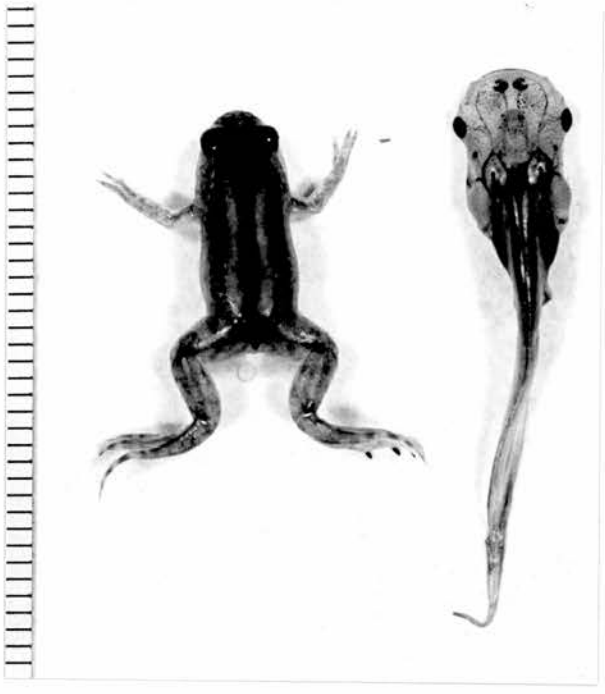


Fig 2

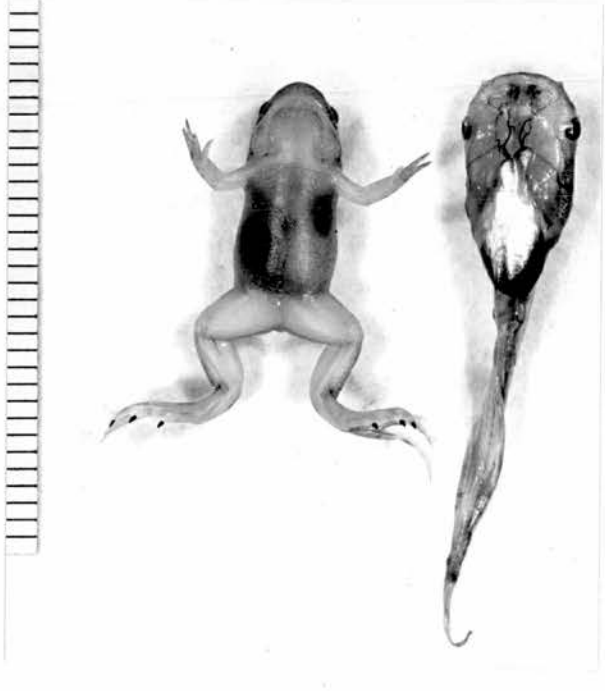
Dorsal aspect of newly metamorphosed frog at Stage 66NF (A) and of tadpole at Stage 57NF (B).
Scale in millimetres.

Fig 3

Ventral aspect of newly metamorphosed frog at Stage 66NF (A) and of tadpole at Stage 57NF (B).
Scale in millimetres.



A B



A B

Fig 4

Cleared whole mount of tadpole lower jaw at Stage 57NF. Meckel's cartilages ▲ are now incompletely fused with the inferior labial cartilage ▼ , the lines where fusion is occurring are still evident ↓ .

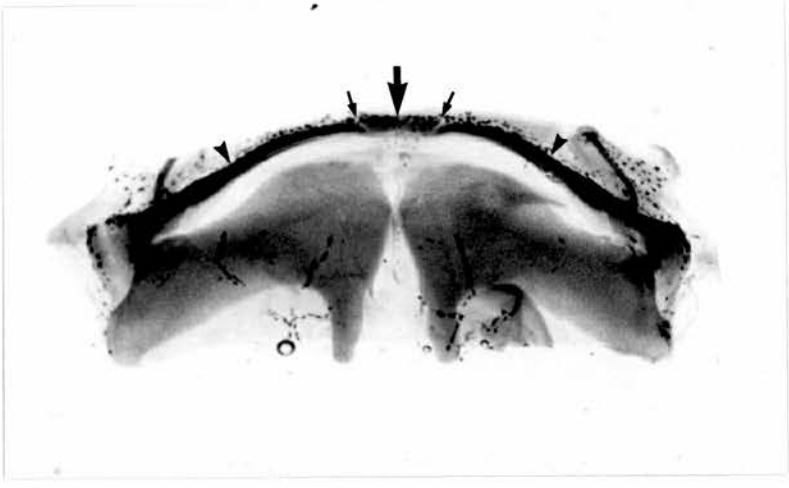
Scale bar = 1mm.

Fig 5

Cleared whole mount of newly metamorphosed frog lower jaw at Stage 66NF.

Meckel's cartilage ▲

Scale bar = 1mm.



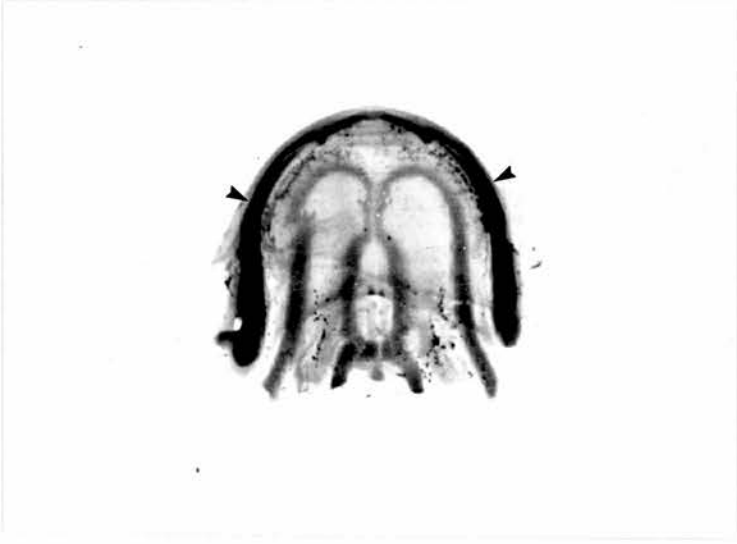


Fig 6

Tissue fixed with 3.125% glutaraldehyde in 0.1M cacodylate buffer.

Peripheral region of a disrupted cell, showing fragmented cell membrane and scattered cytoplasmic remnants.

Stage 57NF

x 21,100

Fig 7

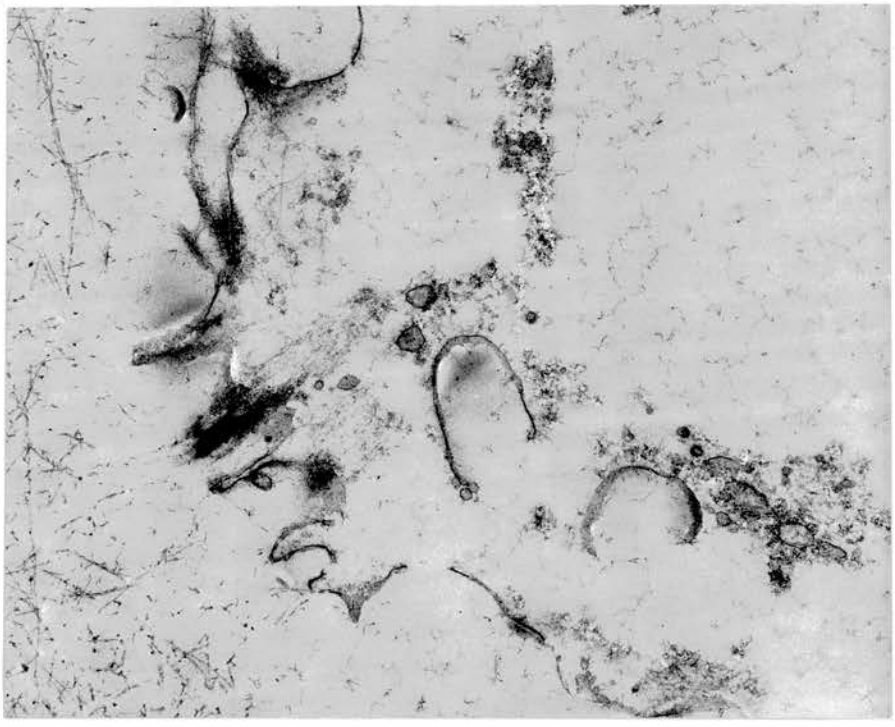
Tissue fixed with 4% paraformaldehyde, 0.5% gluteraldehyde and 0.01% calcium chloride in 0.1% cacodylate buffer.

Part of a cell showing nucleus, cell membrane and disrupted mitochondria.

Stage 57NF

x 23,500

n nucleus
m mitochondria



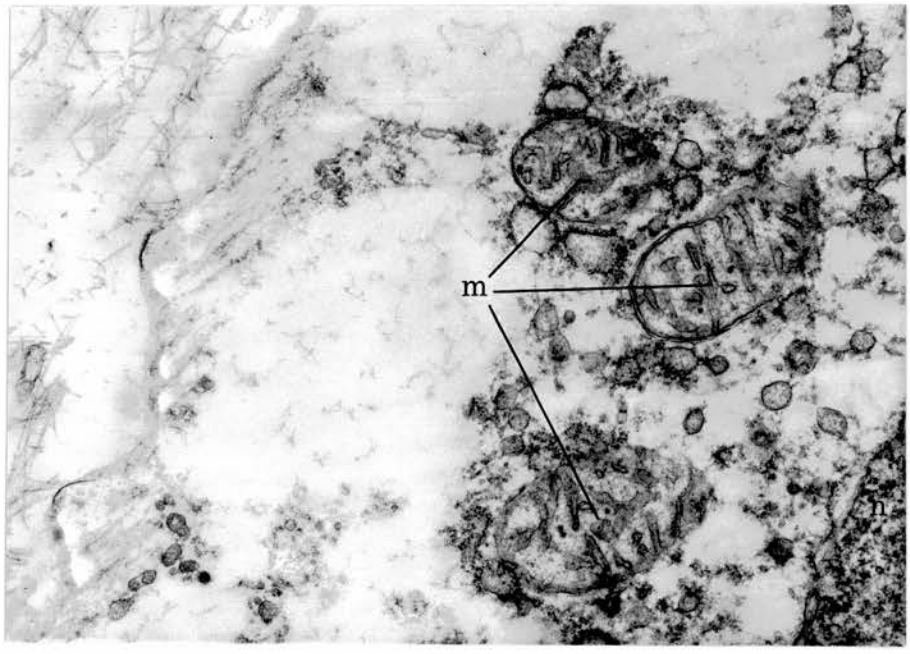

m
n

Fig 8

Tissue fixed in Osmium dissolved in Frog ringer solution, showing grossly swollen mitochondria and poorly fixed cytoplasmic material.

The cell membranes, nuclear envelope and Golgi membranes are satisfactorily preserved.

Stage 57NF

x 16,400

m mitochondria

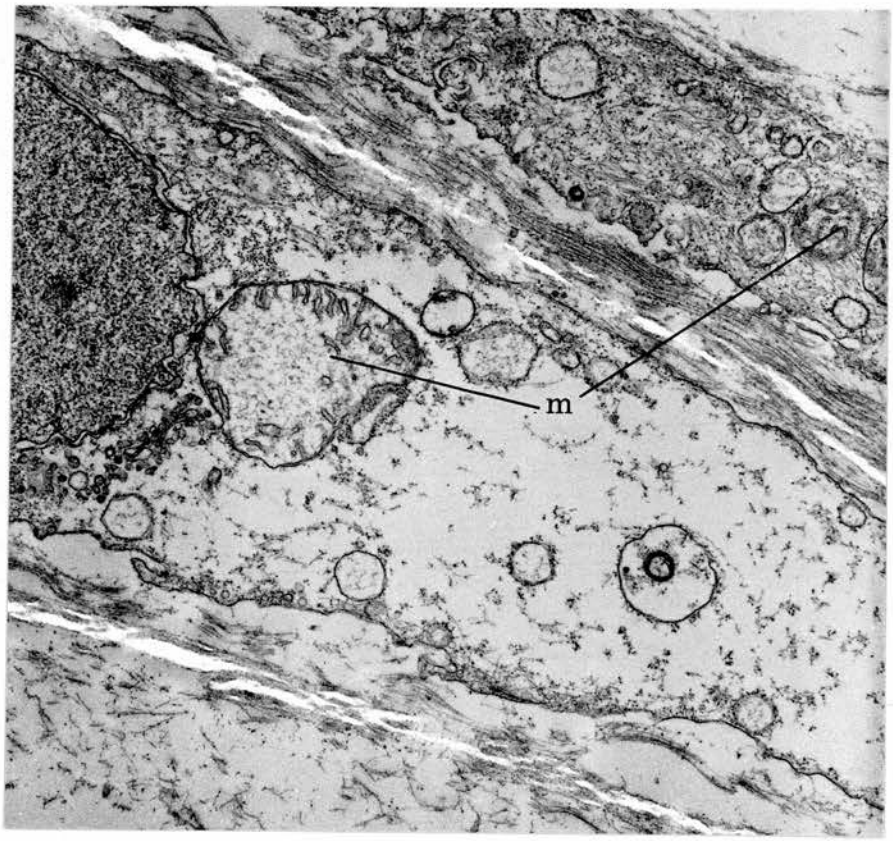

m

Fig 9

Tissue fixed with Osmium dissolved in water with 0.02% calcium chloride added.
Cell membranes and nuclei satisfactorily fixed, but cytoplasmic material poorly fixed and rather diffuse.

Stage 57NF

x 2,600

Fig 10

Tissue fixed as in Fig 9
Part of cell, showing part of nucleus, cell membrane, with disrupted cytoplasm and mitochondria.

Stage 57NF

x 17,800

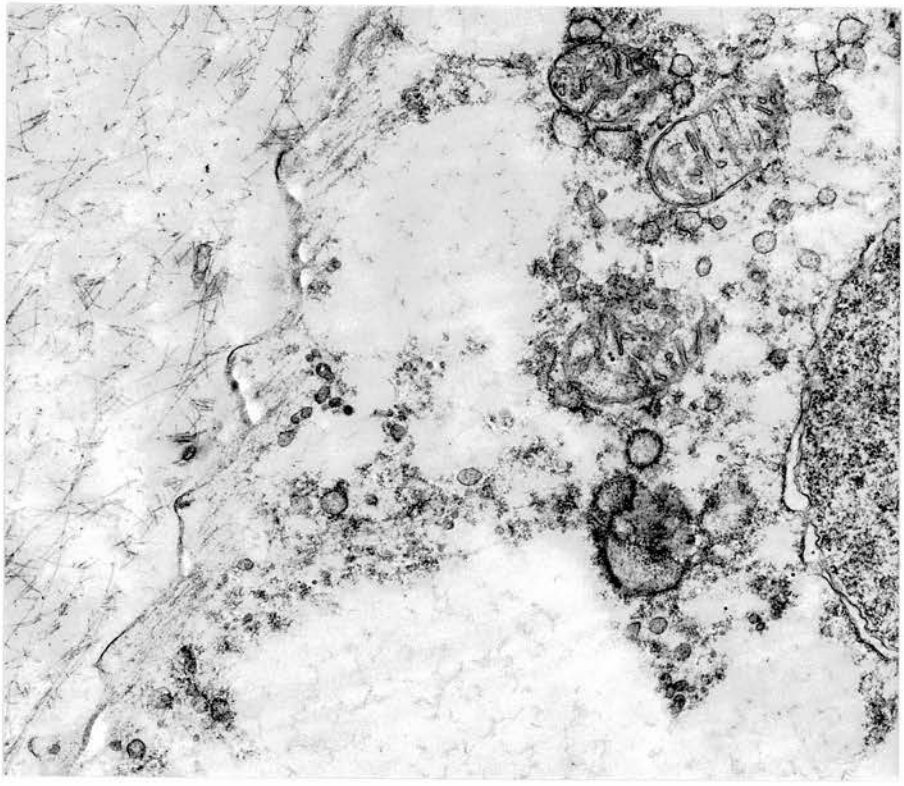


Fig 11

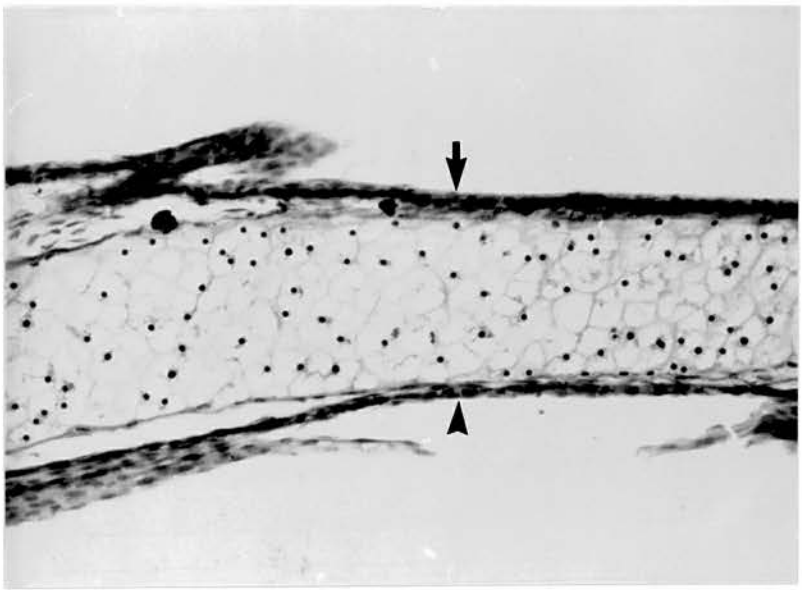
Part of Meckel's cartilage at Stage 57NF, showing large lacunae, with little matrix present. The epidermis ↓ and oral epithelium ▲ lie close against the cartilage.

Horizontal sections x 64

Fig 12

Part of the ceratohyal cartilage at Stage 57NF, showing an appearance similar to that of Meckel's cartilage, with larger lacunae.

Horizontal sections x 64



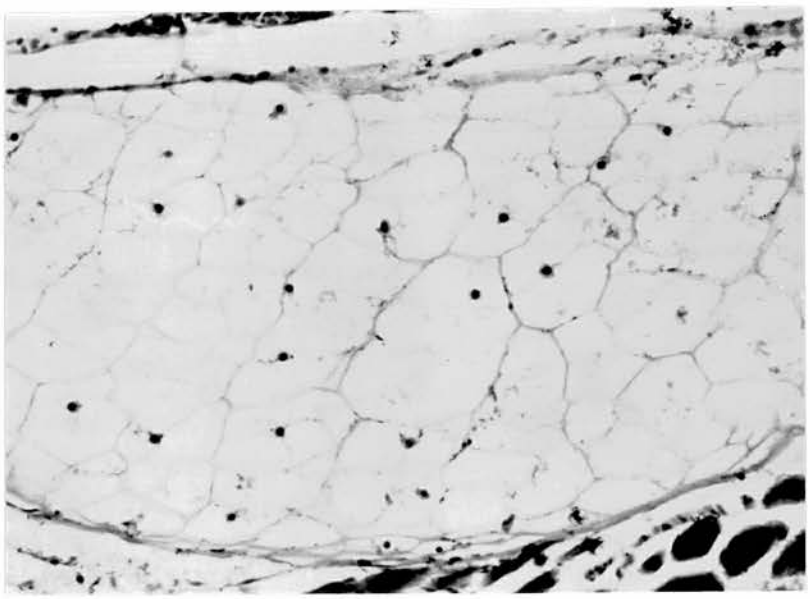


Fig 13

Posterior part of Meckel's cartilage at Stage 57NF, showing its articulation with the quadrate area of the base of the chondrocranium.

Horizontal section x 64

MC Meckel's cartilage

Q quadrate

J joint cavity

↑ joint capsule

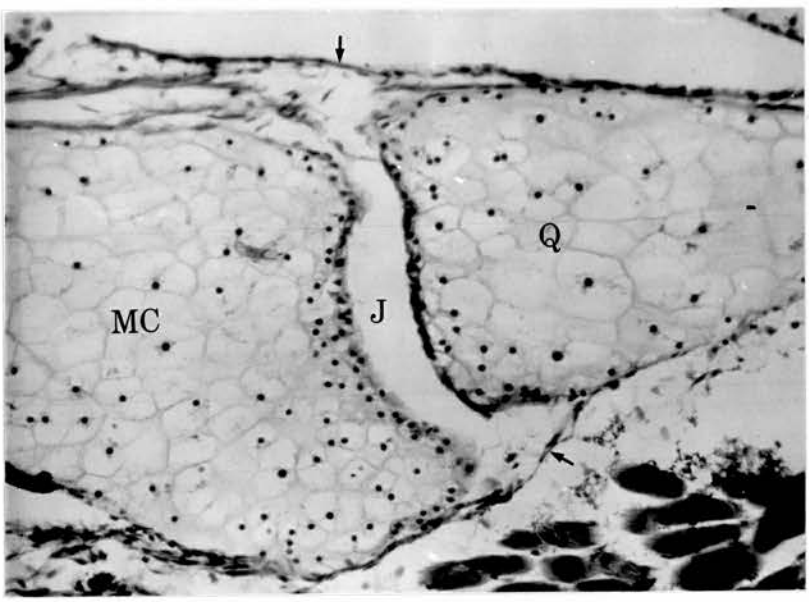

MC
J
Q

Fig 14

Part of Meckel's cartilage at Stage 60NF, showing the goniale beginning to ossify, and increased width of lamina propria.

Horizontal section x 64

Fig 15

Part of Meckel's cartilage at Stage 63NF, showing a slight increase in the amount of matrix present.

Horizontal section x 64

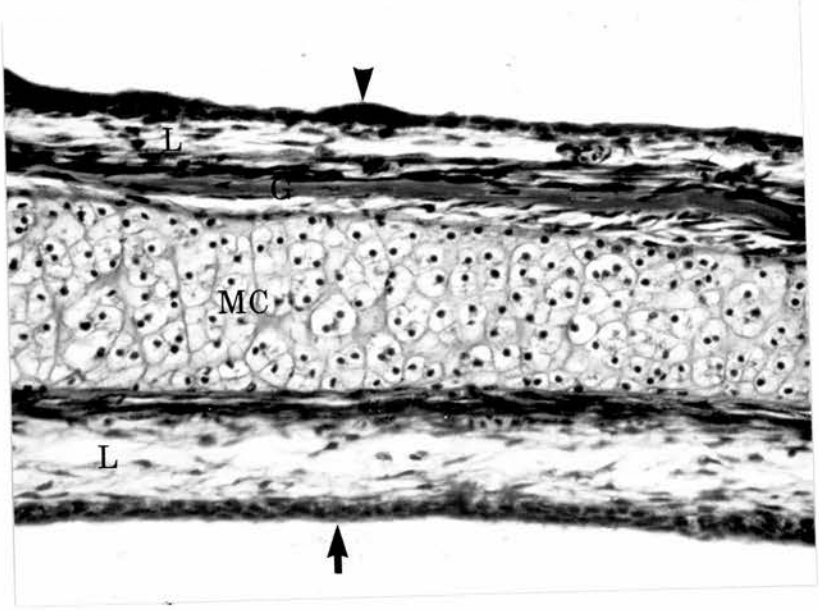
MC Meckel's cartilage

G bone of goniale

L lamina propria

▲ oral epithelium

▲ epidermis


L
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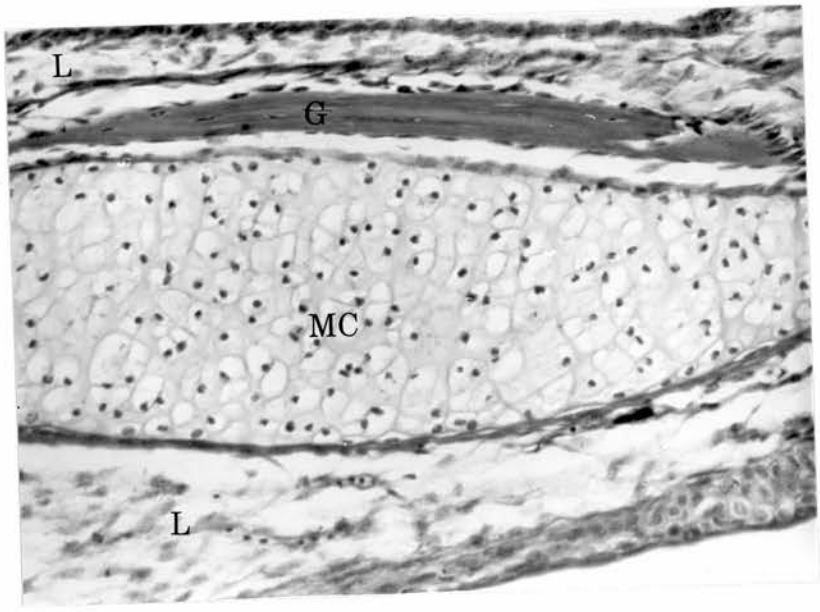
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Fig 16

Anterior part of Meckel's cartilage at Stage 66NF, near the midline, showing smaller lacunae and a considerably increased amount of matrix present.

Horizontal section x 64

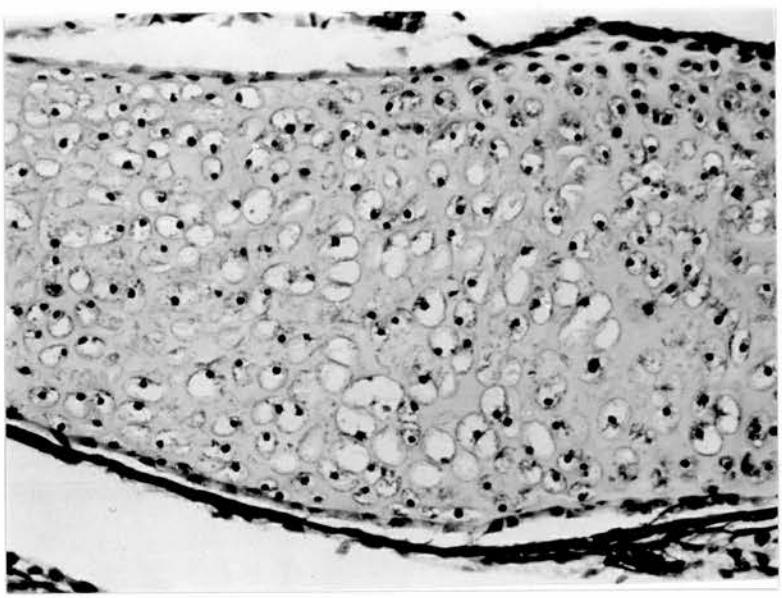


Fig 17

Posterior part of Meckel's cartilage at Stage 66NF showing the goniale and dentale ossifying around it.

Horizontal section x 64

Fig 17A

Posterior part of Meckel's cartilage at Stage 66NF showing the goniale and dentale.

Transverse section

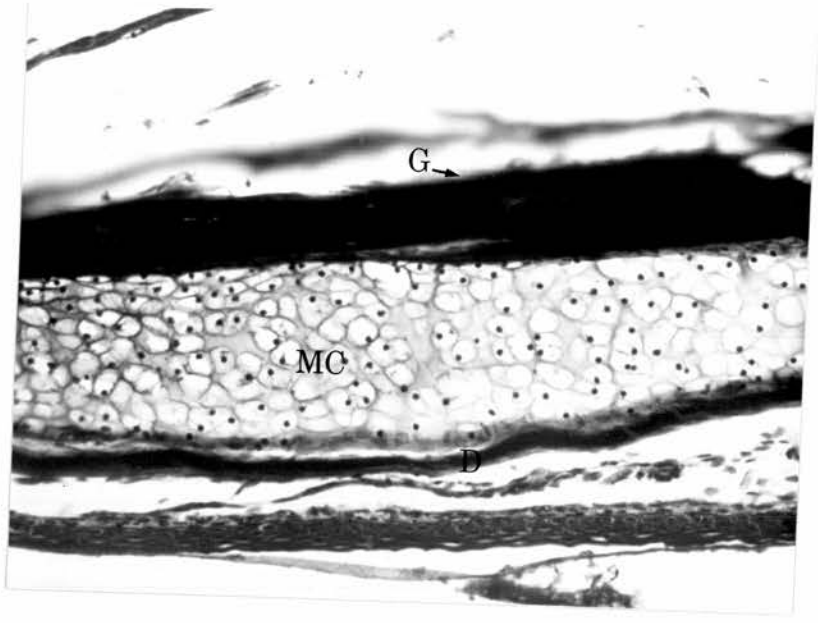
MC Meckel's cartilage

G goniale

D dentale

▲ oral epithelium

m lower jaw musculature


G
MC
D

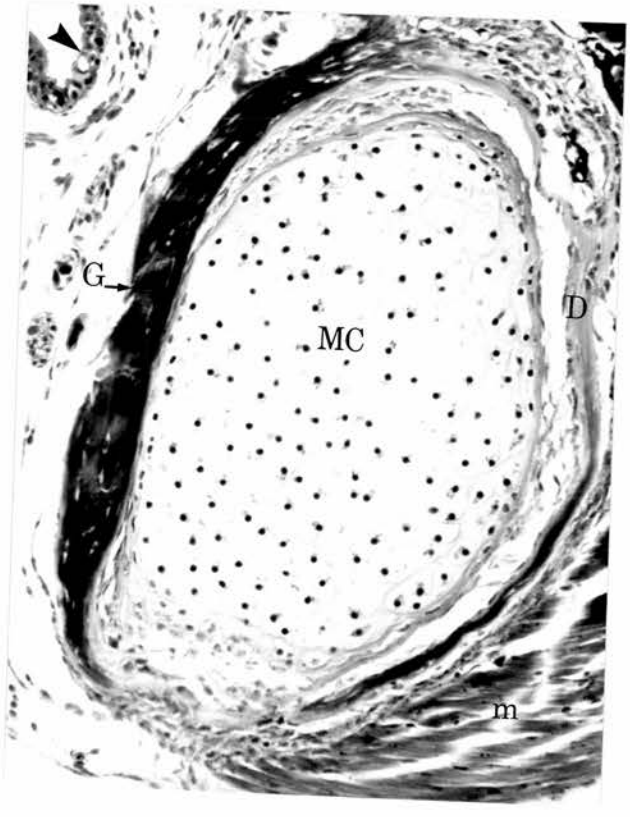

G
MC
D
m

Fig 18

Posterior part of Meckel's cartilage at Stage 66NF showing its articulation with the quadrate area of the base of the chondrocranium. The high cell density in both cartilages beneath the articular surfaces is evident.

Horizontal section x 64

MC Meckel's cartilage

Q quadrate

J joint cavity

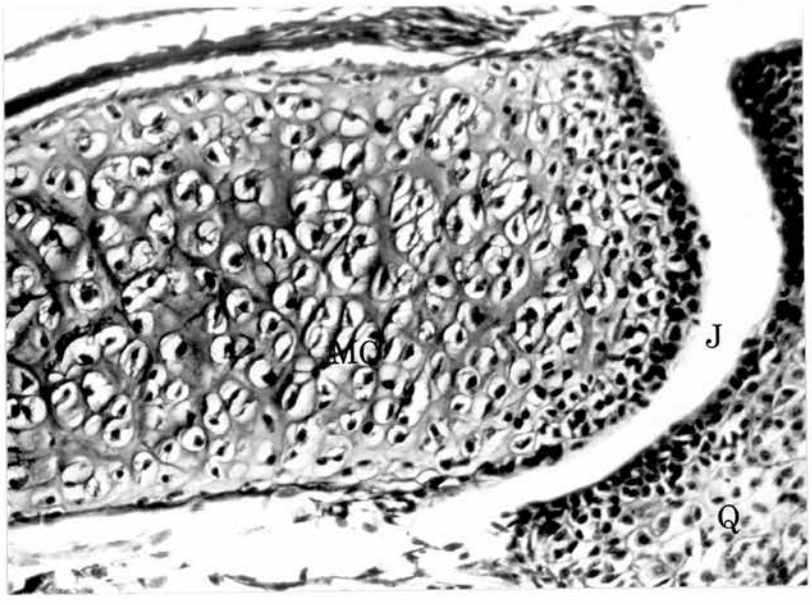

MC
J
Q

Fig 19

Part of the ceratohyal cartilage at Stage 66NF.
The lacunae are still relatively large, and the amount of matrix present low.

Horizontal section x 64

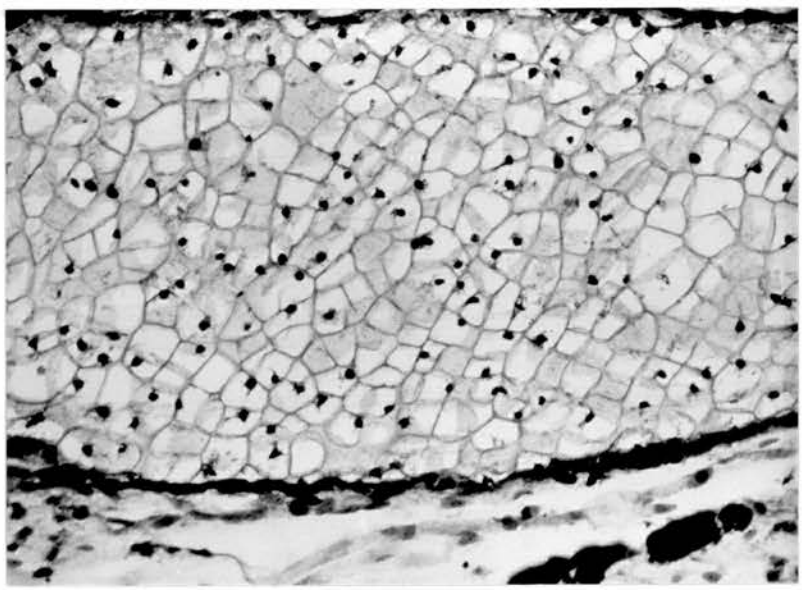


Fig 20

Early metamorphosis - peripheral zone cells.

x 4,210

f perichondrium, part of a fibroblast

p peripheral, flattened cells

d deeper, spheroidal cells

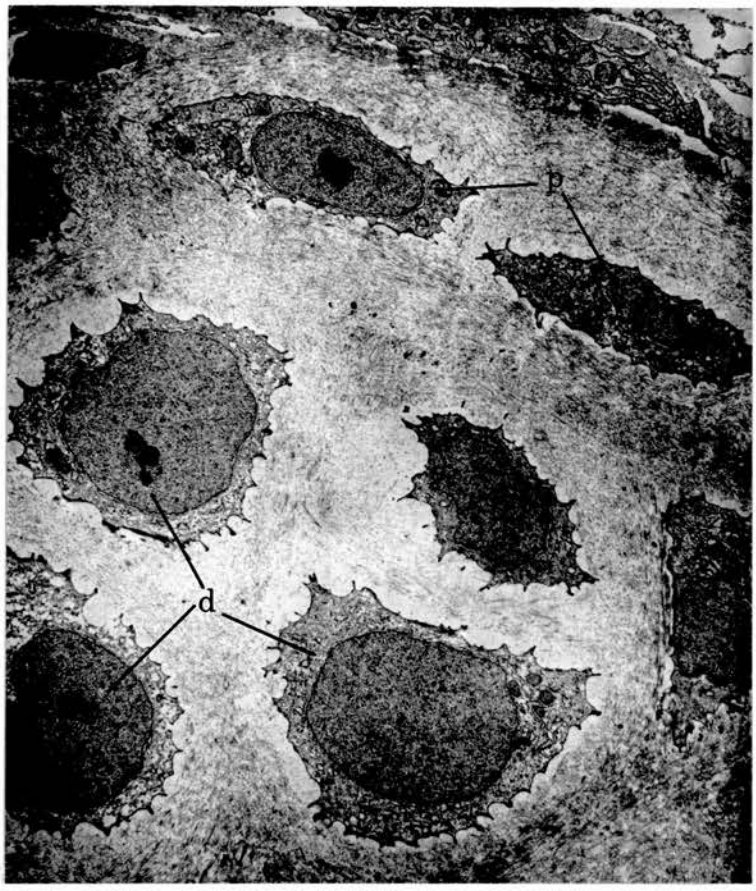

p
d

Fig 21

Early metamorphosis - part of a peripheral cell.

x 24,300

Fig 22

Early metamorphosis - part of a peripheral cell.

x 29,800

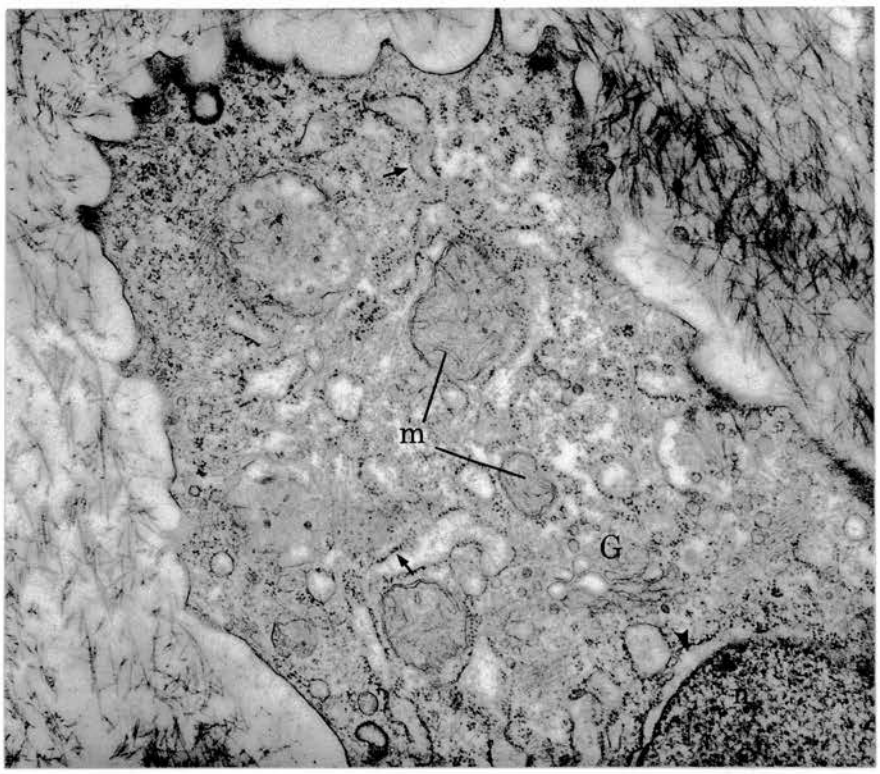
n nucleus

▲ perinuclear cistern

G Golgi complex

m mitochondria

r granular endoplasmic reticulum


m
G
n

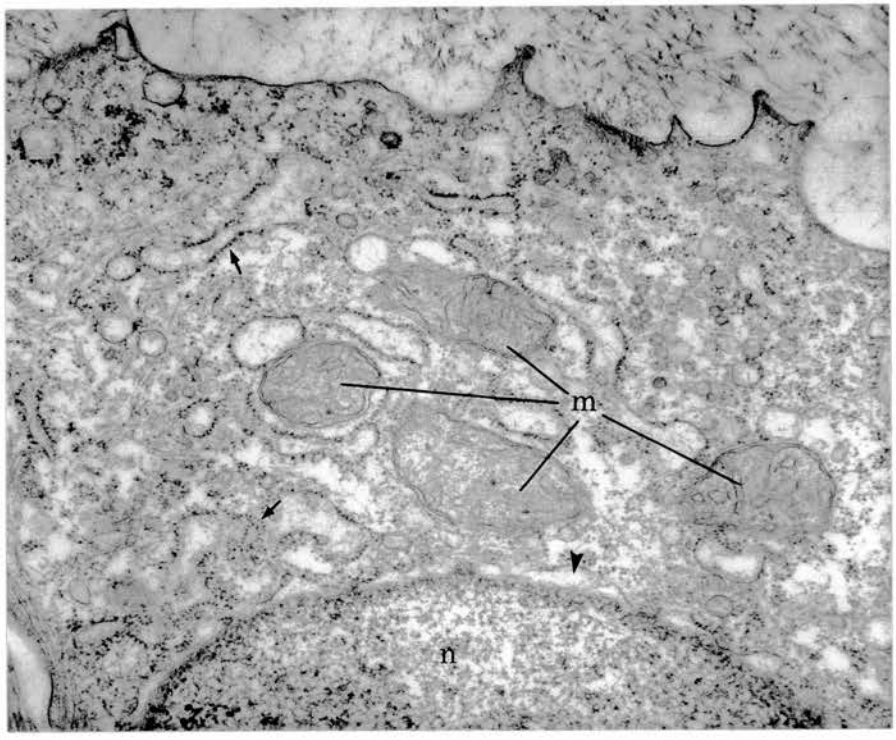

m
n

Fig 23

Early metamorphosis - deeper, spheroidal cell.

x 9,300

n nucleus

o nucleolus

↑ granular endoplasmic reticulum

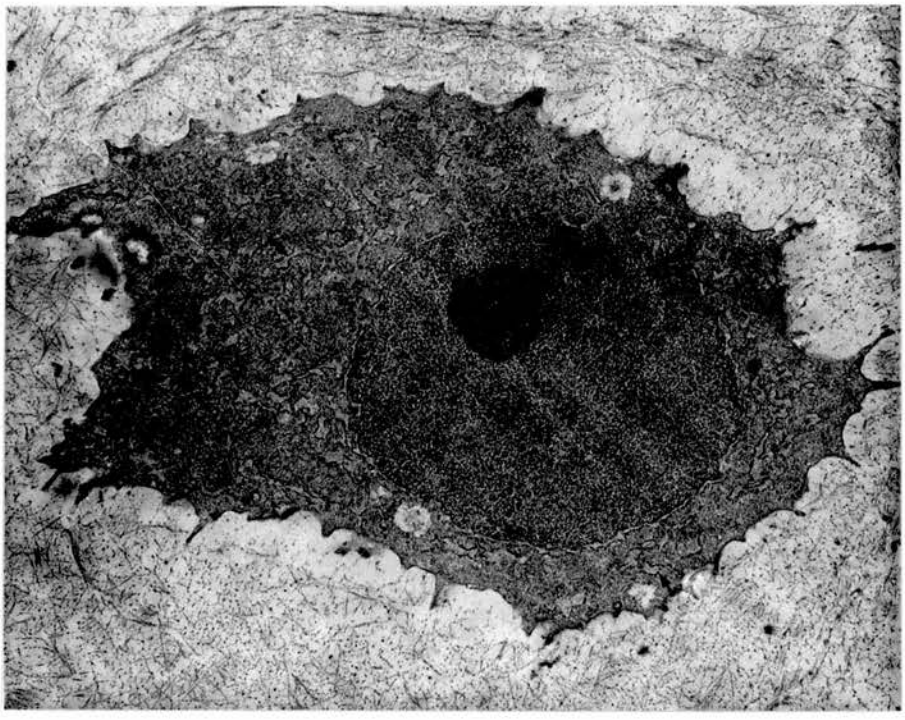


Fig 24A

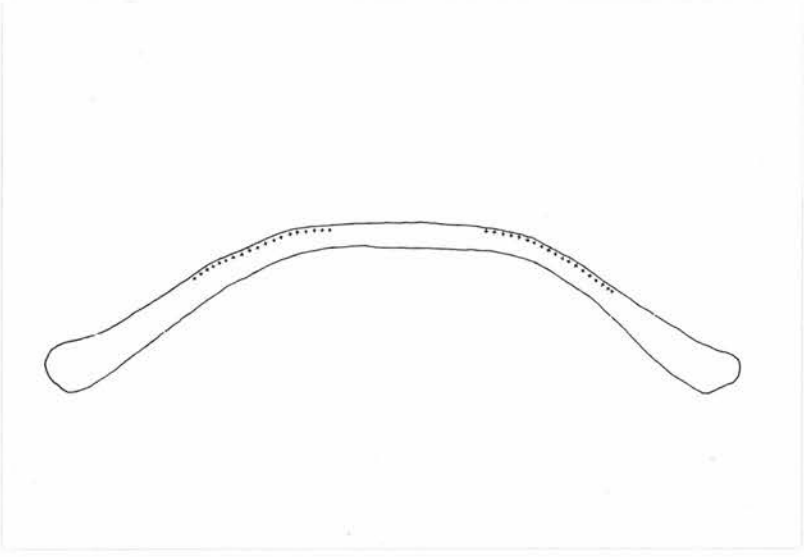
Diagrammatic horizontal section of Meckel's cartilage during early metamorphosis showing zones of cell columns +++

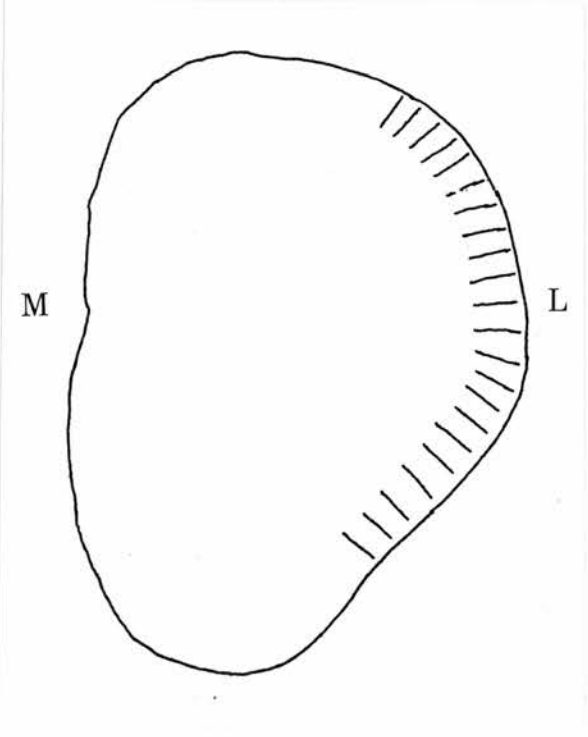
Fig 24B

Diagrammatic transverse section of Meckel's cartilage during early metamorphosis, showing the distribution of the cell columns.

M medial

L lateral




M
L

Figs 25 & 26

Early metamorphosis - cell columns from Meckel's cartilage.

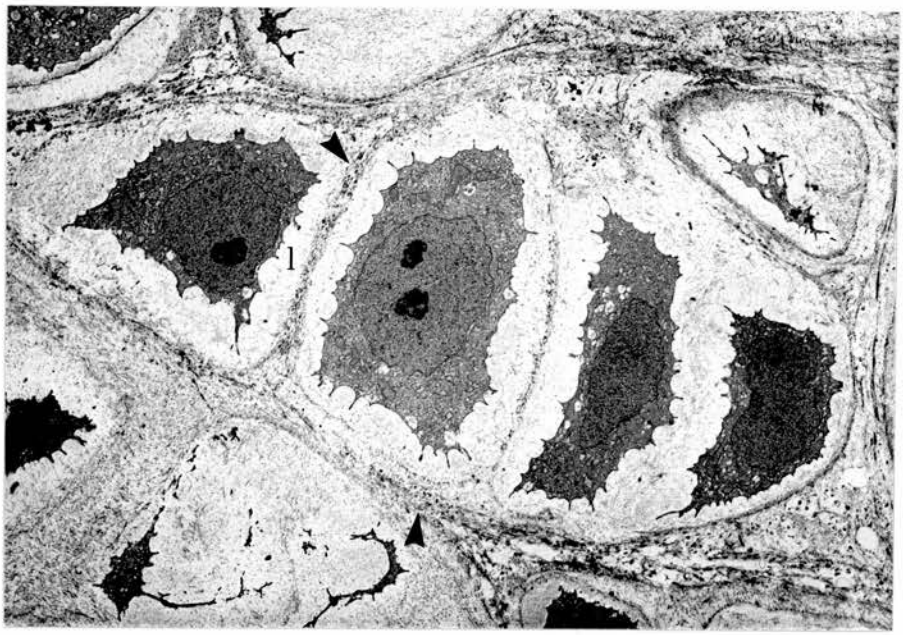
x 2,940

l lacunar matrix

▲ interlacunar matrix

G Golgi complex

➡ periphery


1

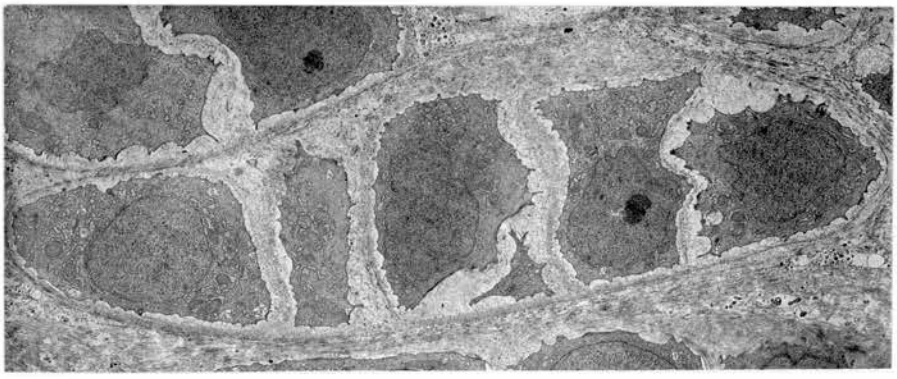


Fig 27

Early metamorphosis - two recently divided cells, with a rim of interlacunar matrix developing between them.

x 4,560

l lacunar matrix

▲ interlacunar matrix

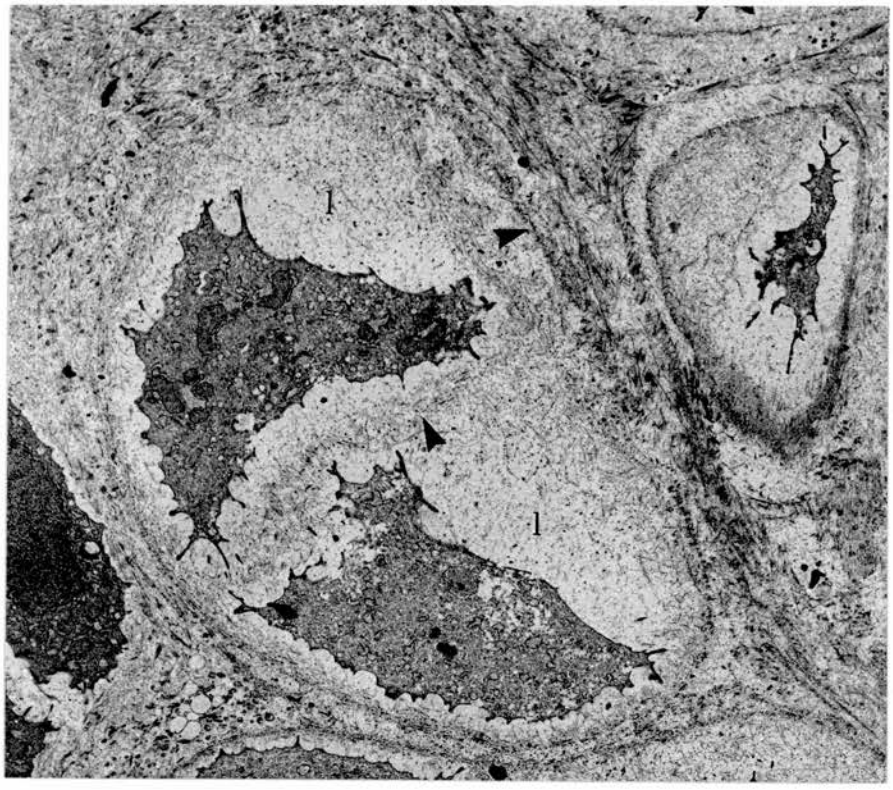


Fig 28

Early metamorphosis - cell below proliferative zone.

x6,150

G Golgi complex with vacuoles

vacuoles apparently discharging material

↑ into the lacunar matrix

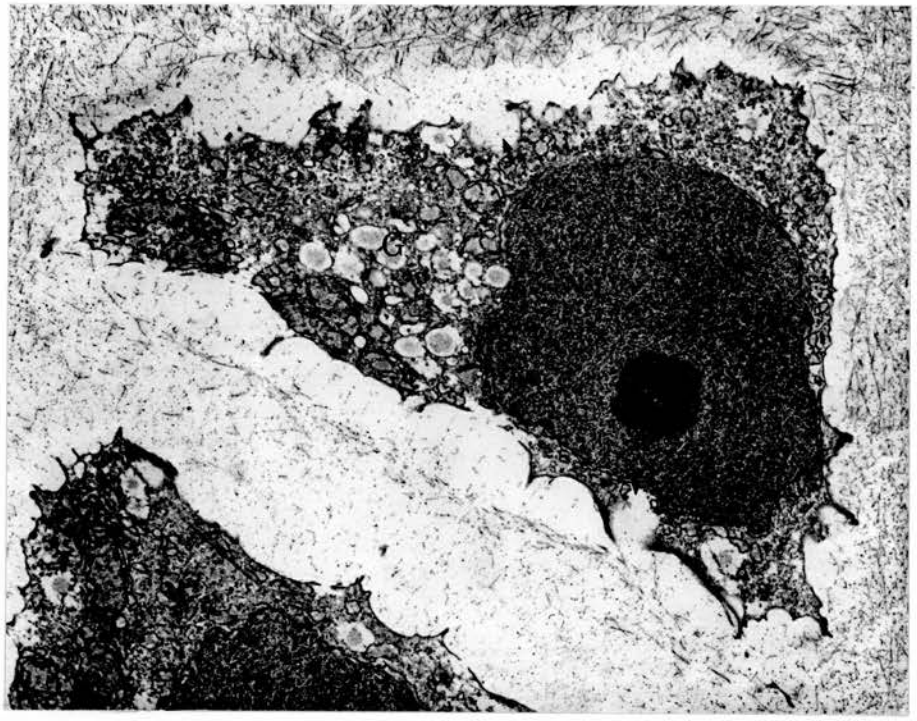


Fig 29

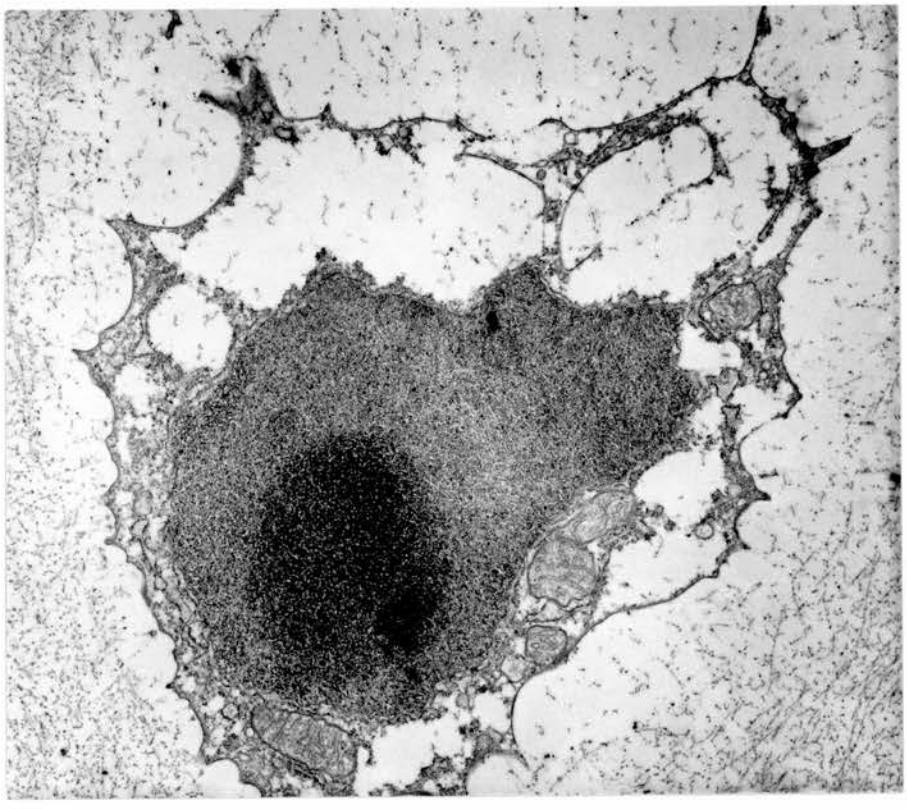
Early metamorphosis - cell from the deepest zone of the cartilage, showing fragmentary cytoplasm, and areas of loosely aggregated material, similar to the lacunar matrix.

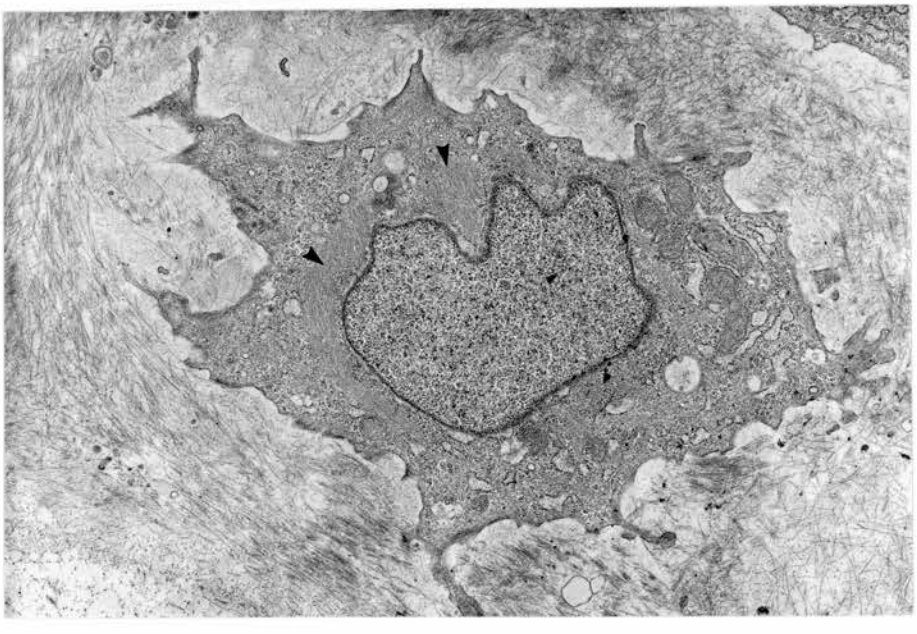
x 11,400

Fig 30

Early metamorphosis - cell from the deepest zone of the cartilage showing an irregularly shaped nucleus and numbers of peri-nuclear fibrils. ▲

x 8,550





Figs 31 & 32

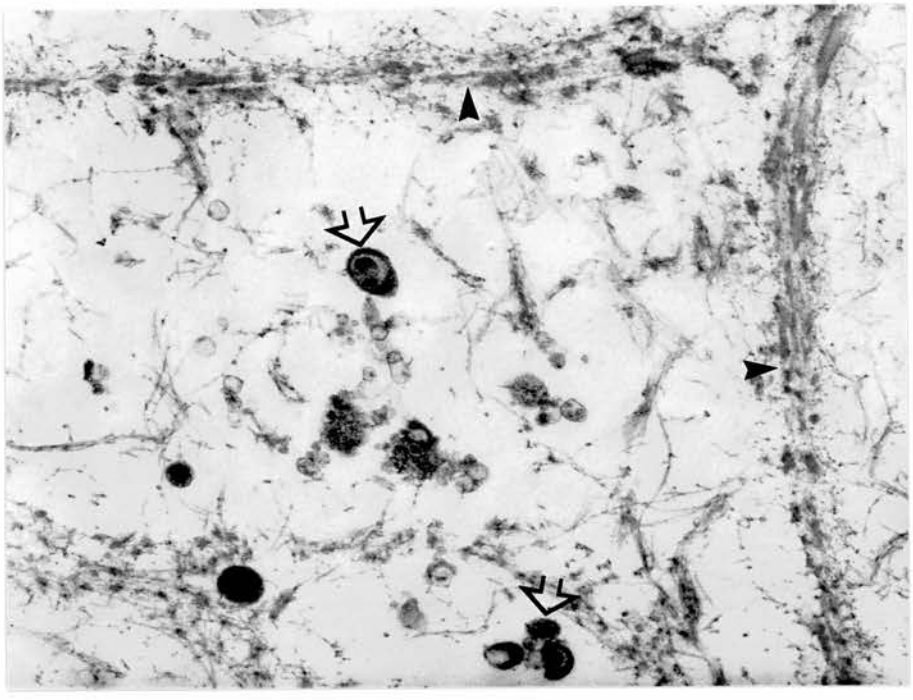
Early metamorphosis - interlacunar areas, showing thin rims of interlacunar matrix with electron dense bodies lying in the lacunar zones.

Fig 31 x 24,800

Fig 32 x 55,200

▲ interlacunar matrix

⇩ dense bodies



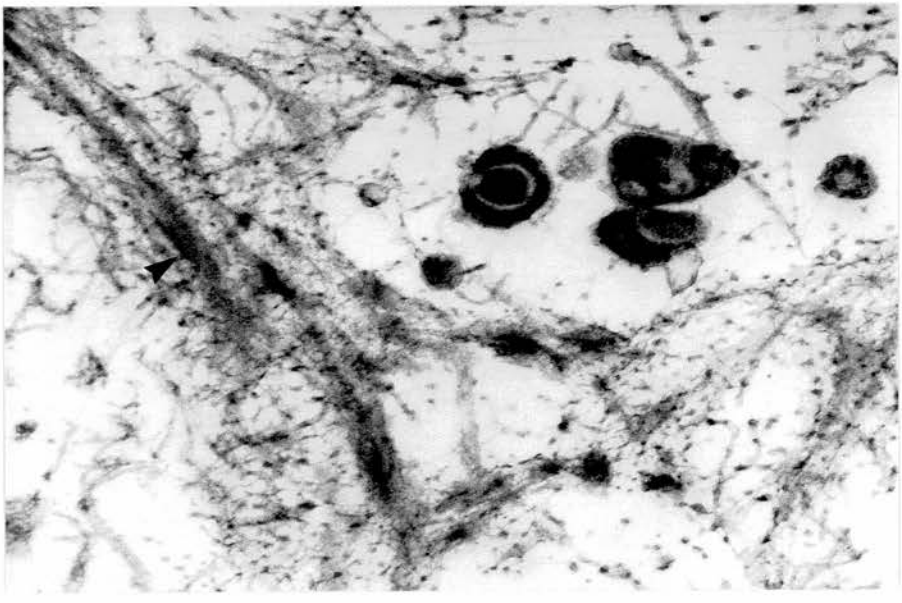


Fig 33

Stage 63NF - peripheral zone cells, showing condensation of fibrils in the interlacunar matrix

perichondrium

x 3,850

Fig 34

Stage 63NF - part of the perichondrium, showing fibroblasts and bundles of fibres, and peripheral cartilage cells.

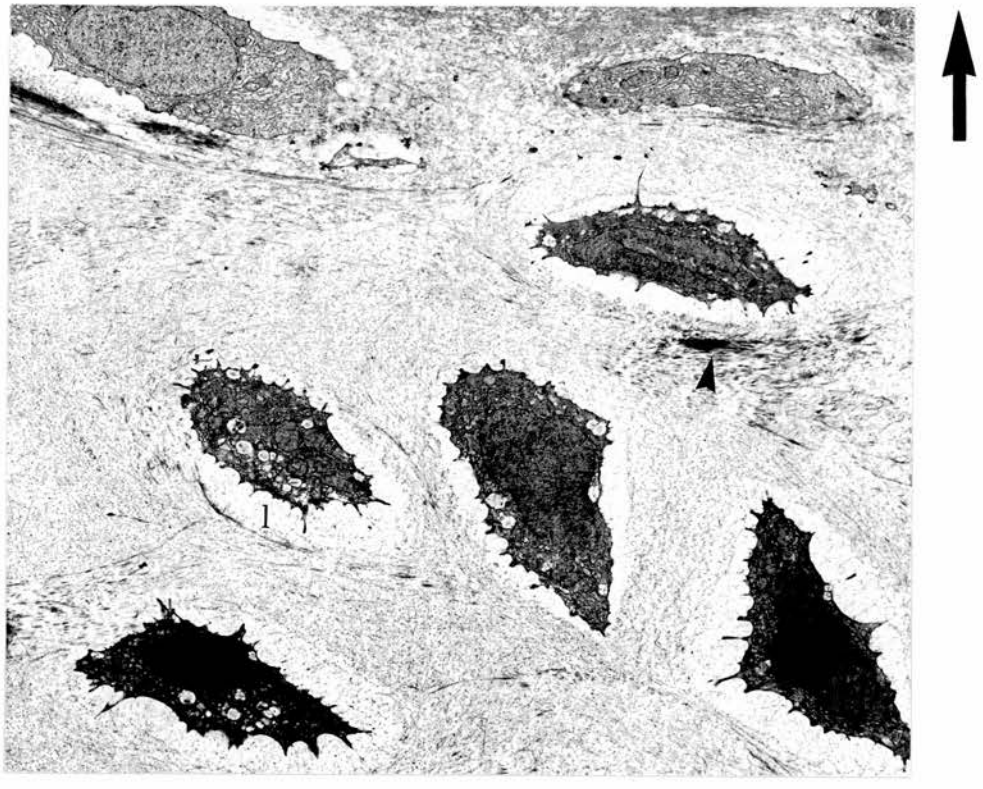
x 4,490

l lacunar matrix

▲ interlacunar matrix, with condensing fibrils

f fibroblasts

 fibre bundles


l

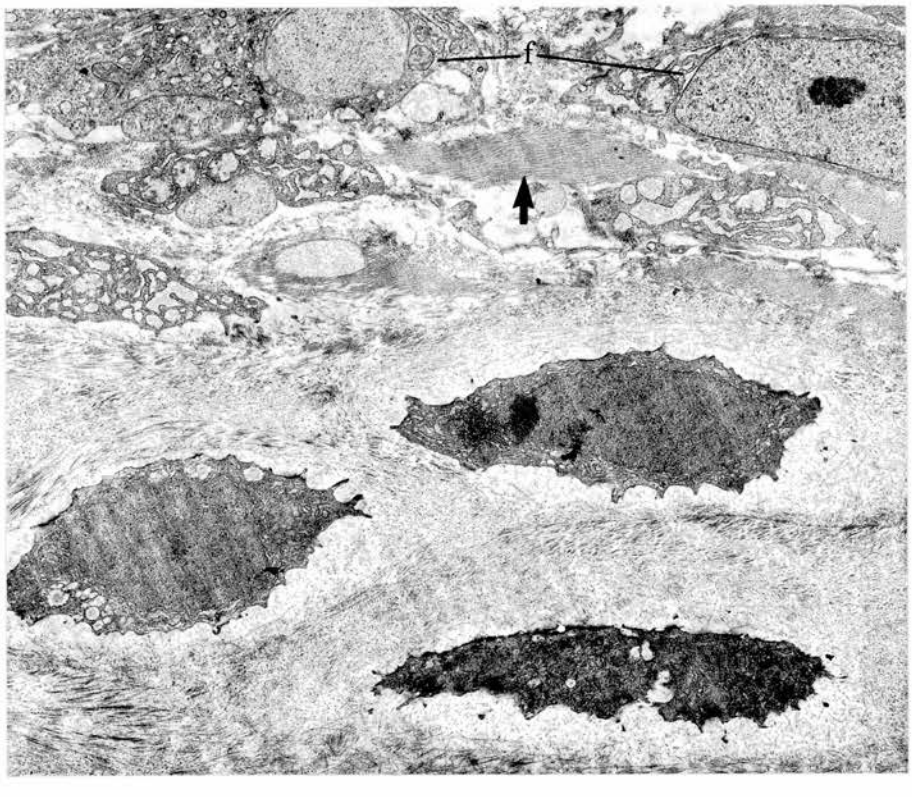

f

Fig 35

Stage 63NF - deeper zone cell.

x 8,980

Fig 36

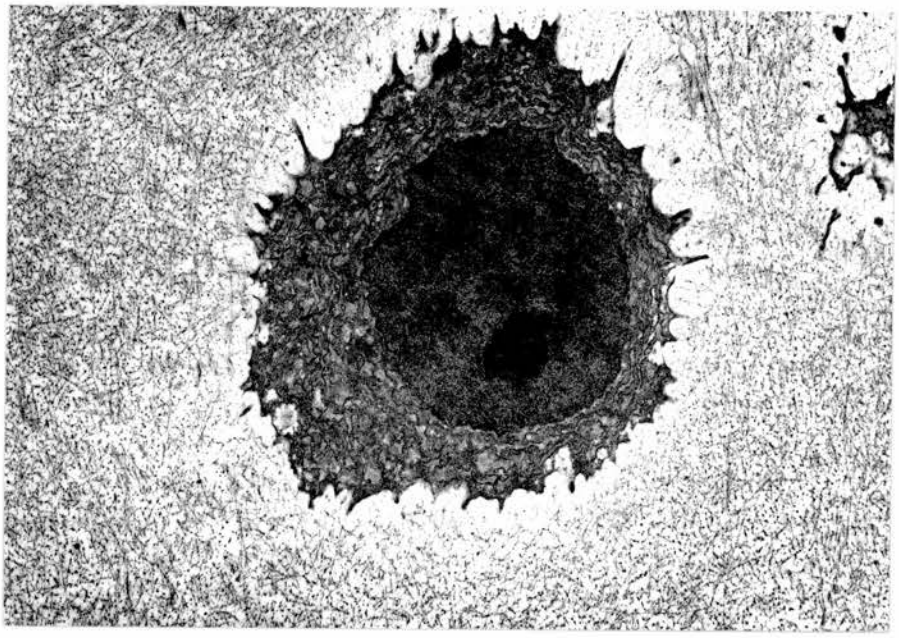
Stage 63 NF - part of a deeper zone cell.

x 9,670

↑ granular endoplasmic reticulum

m mitochondria

G Golgi vacuoles



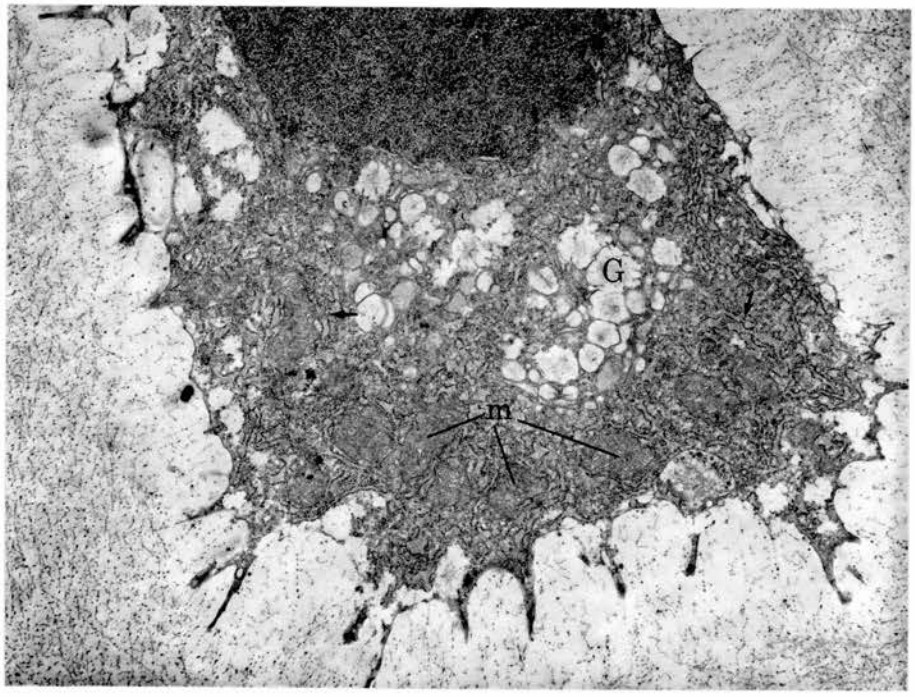

G
m

Fig 37

Stage 63NF - deeper zone cell showing a single aberrant cilium. ↑

x 15,900

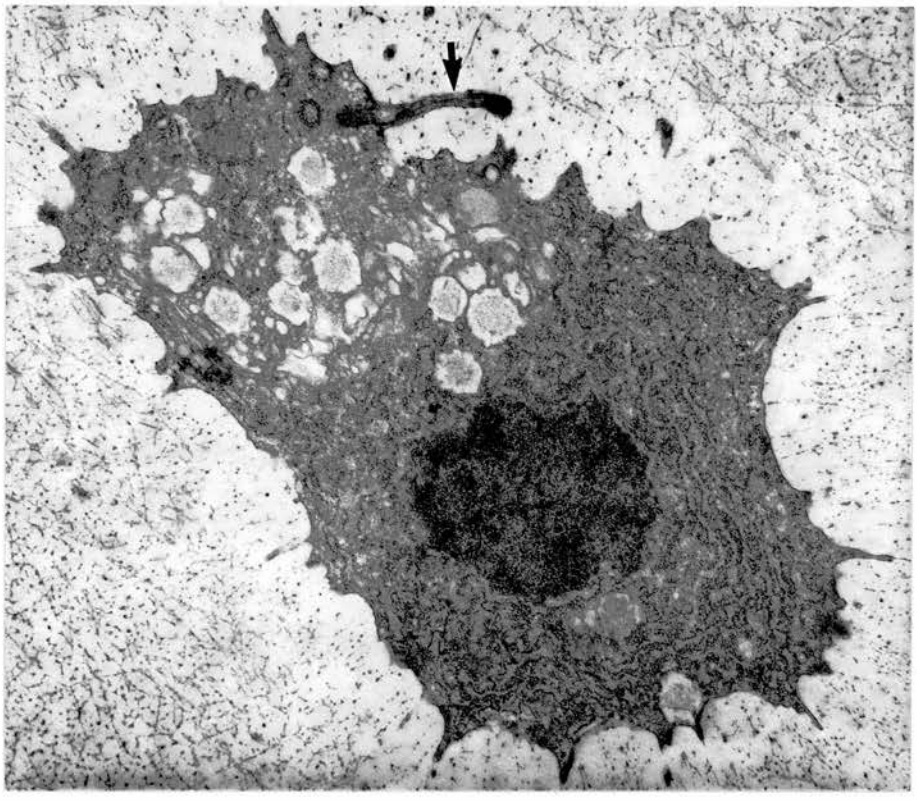
Fig 38

Stage 63NF - part of the deeper zone cell shown in Fig 37, showing the base of the cilium, accompanied by a second centriole.

x 39,700

b base of cilium

c centrioles



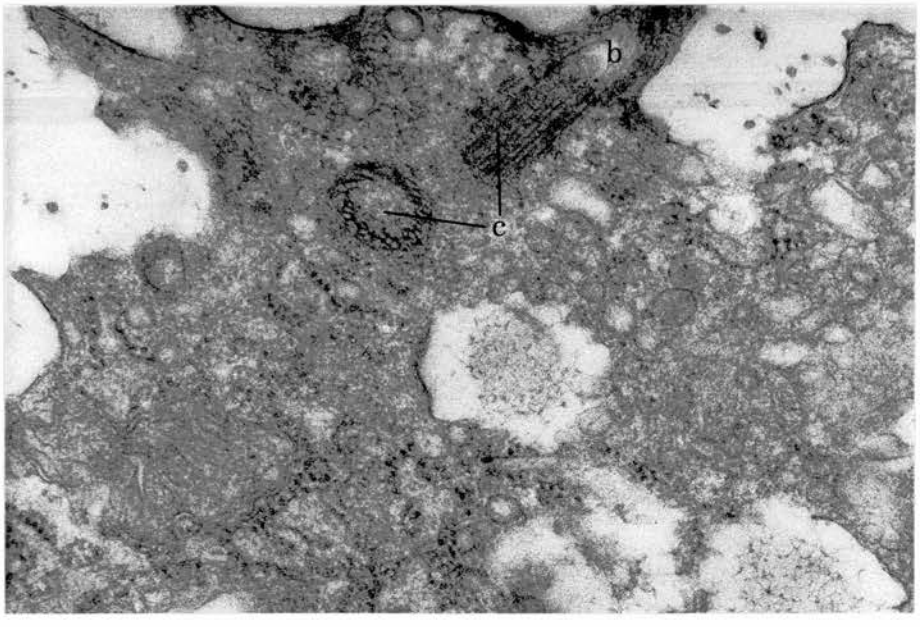

b
c

Fig 39

Stage 66NF - peripheral zone of Meckel's cartilage.

x 2,440

p perichondrium, with parts of fibroblasts

c cartilage cells

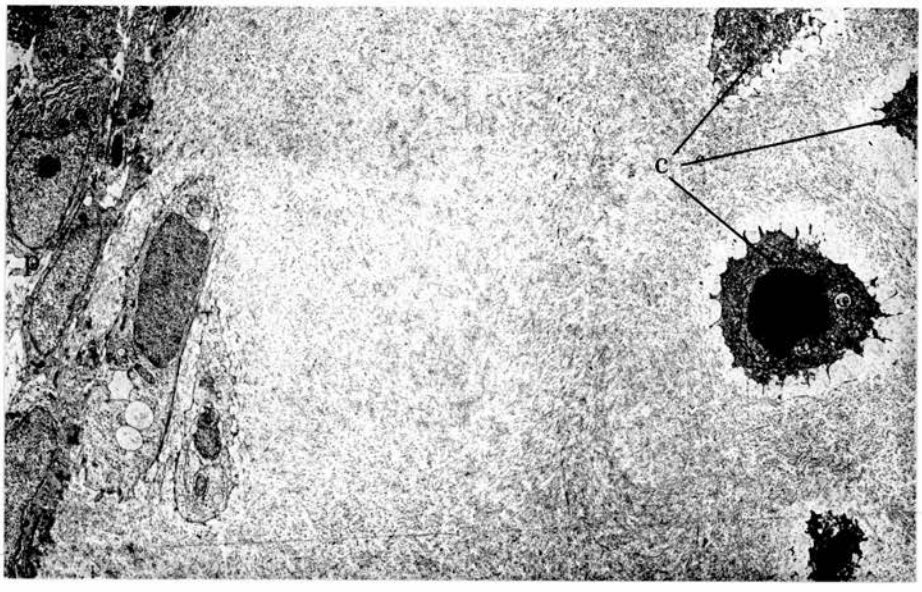

C
P

Fig 40

Stage 66NF - cells below peripheral zone.

x 4,080

Fig 41

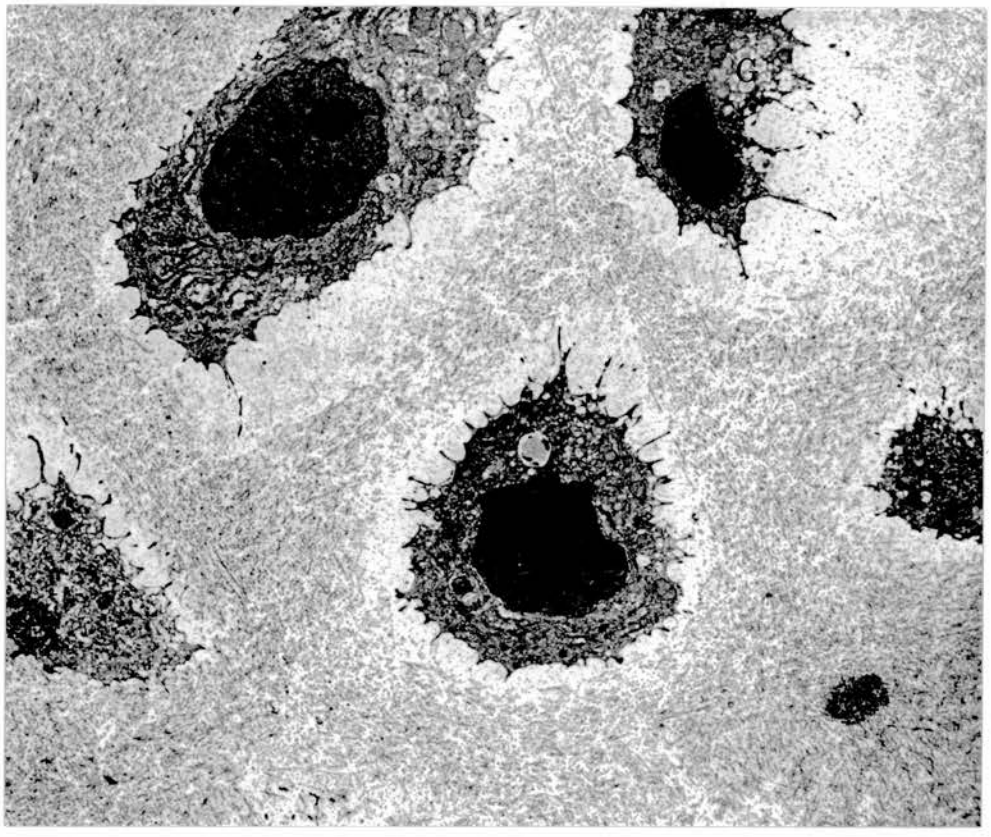
Stage 66NF - cell from subperipheral zone.

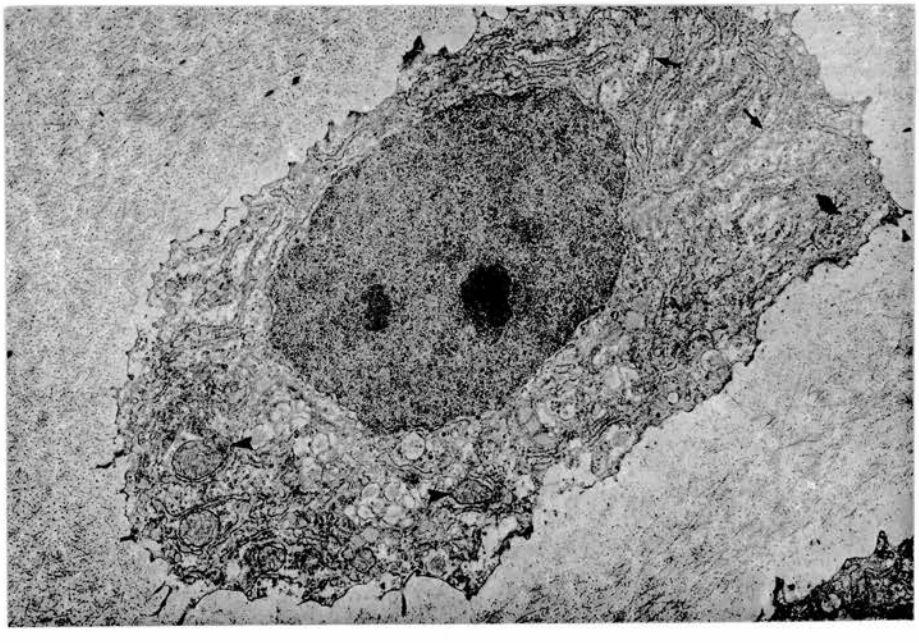
x 6,950

↑ granular endoplasmic reticulum

▲ mitochondria

G Golgi vacuoles


G

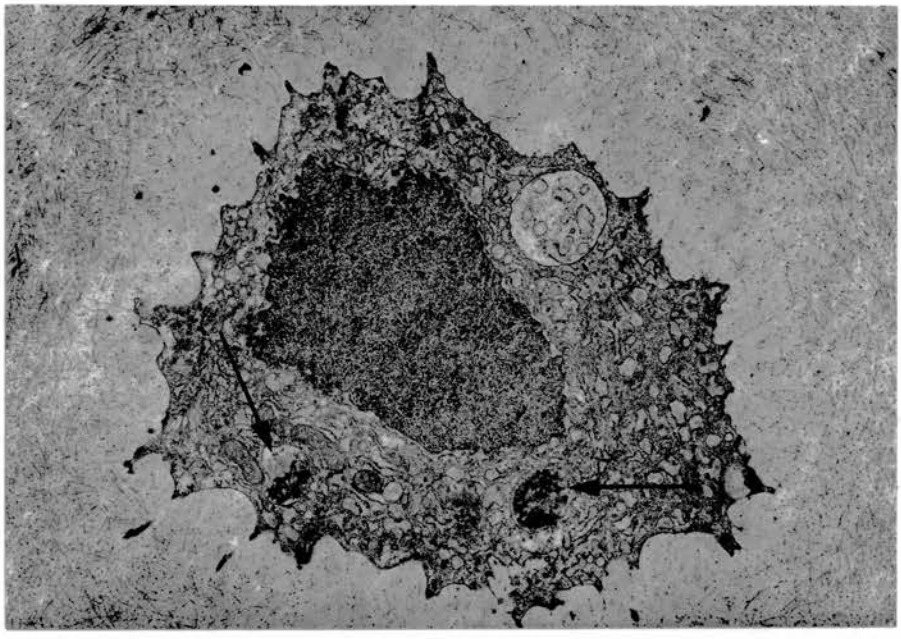


Figs 42 & 43

Stage 66NF - cells showing lysosomes.

Fig 42 x 8,980

Fig 43 x 9,620



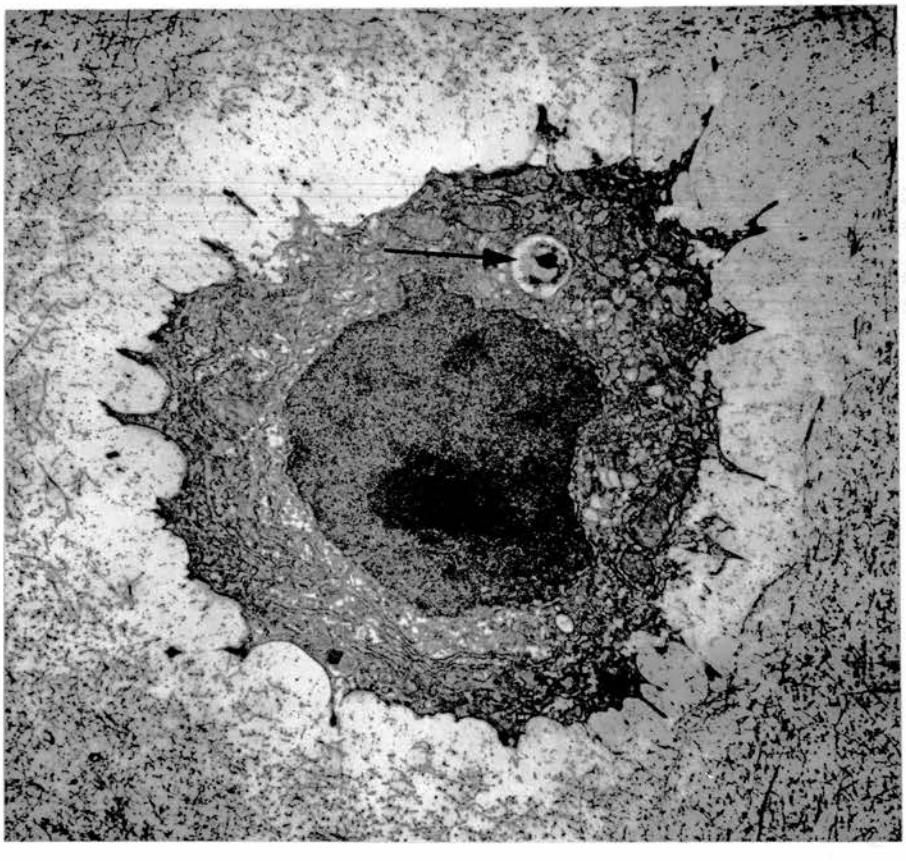


Fig 44

Stage 66NF - deep zone, showing a degenerating cell with fragmenting cytoplasm.

x 7,020

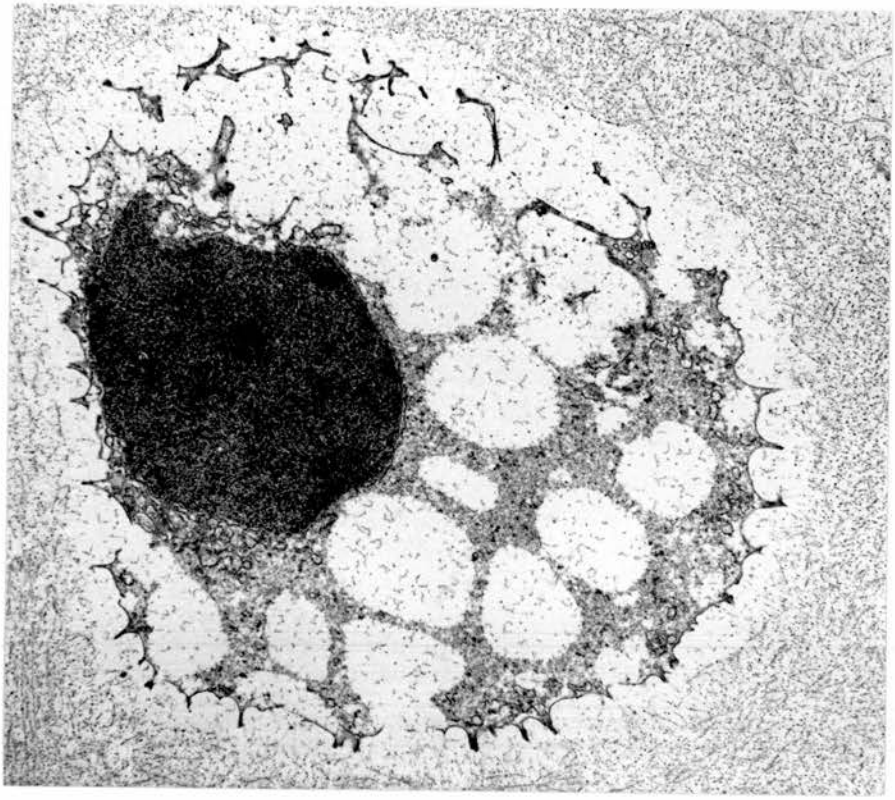


Fig 45

Histogram showing corrected mean total nuclear number ($x \frac{1}{1000}$) for each NF Stage during prometamorphosis and metamorphic climax.

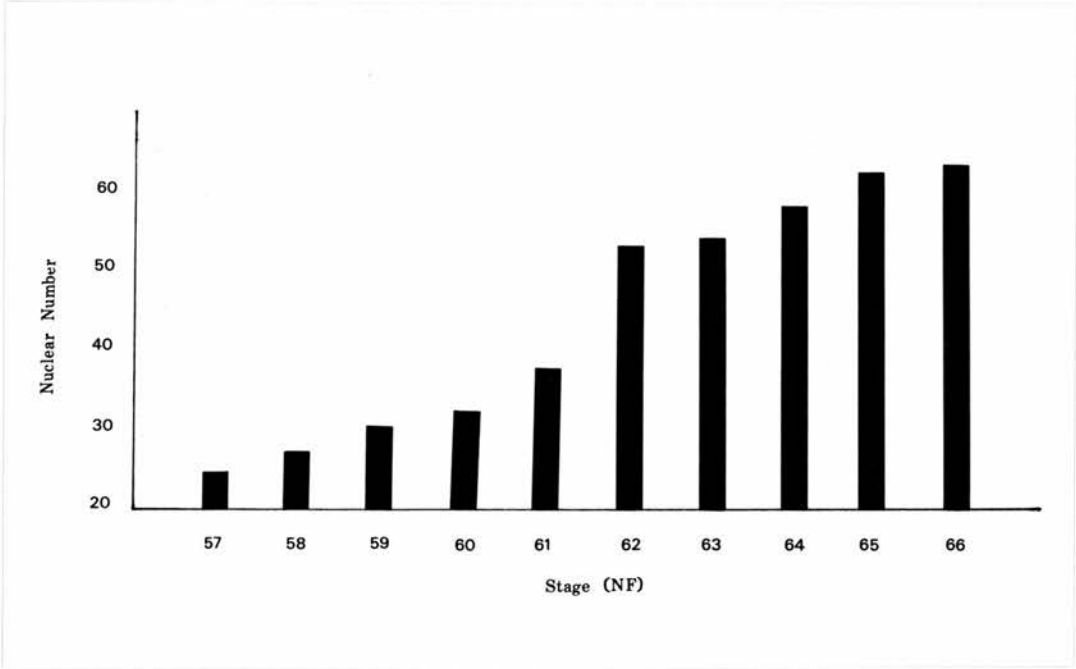

Nuclear Number
60
50
40
30
20
57
58
59
60
61
62
63
64
65
66
Stage (NF)

Fig 46

Histogram showing corrected mean total nuclear number ($x \frac{1}{1000}$) for each NF Stage during prometamorphosis and metamorphic climax, together with the time scale in days post-fertilisation (at 25°C) and the developmental phases.

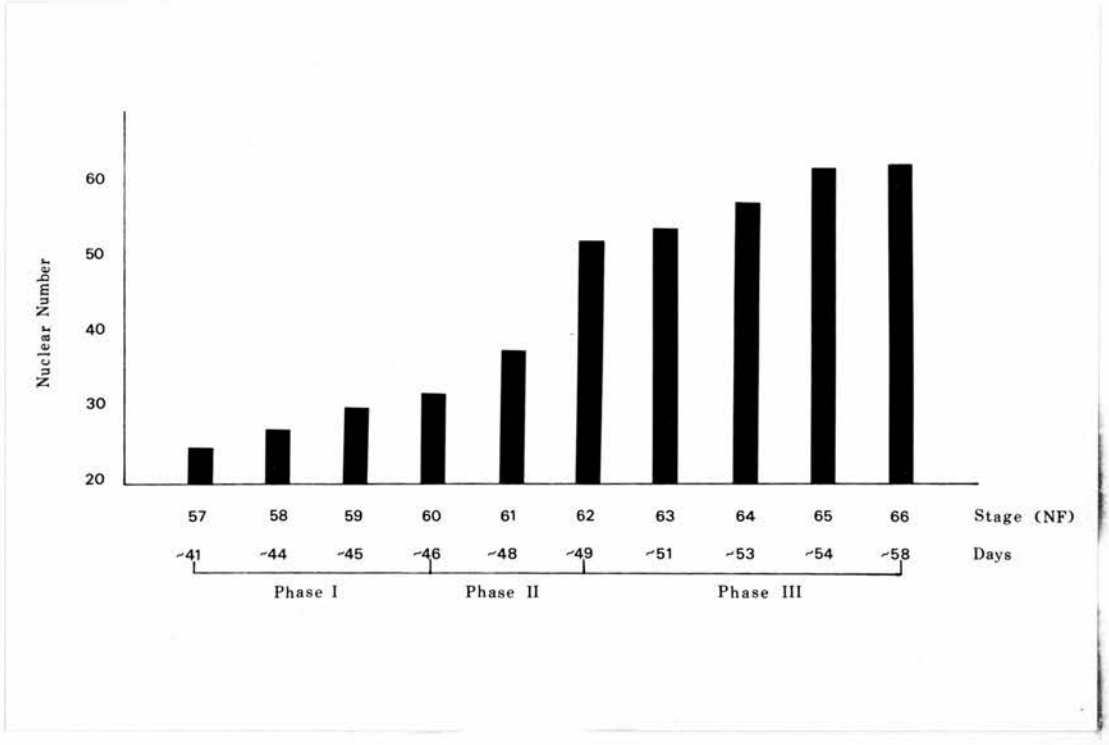

Nuclear Number
60
50
40
30
20
57
58
59
60
61
62
63
64
65
66
Stage (NF)
~41
~44
~45
~46
~48
~49
~51
~53
~54
~58
Days
Phase I
Phase II
Phase III

Fig 47

Graph showing corrected mean nuclear number at each level below the articular surface of Meckel's cartilage at Stages 57, 60, 63 and 66NF.

- ● Stage 57NF
- □ Stage 60NF
- ◇ Stage 63NF
- ○ Stage 66NF

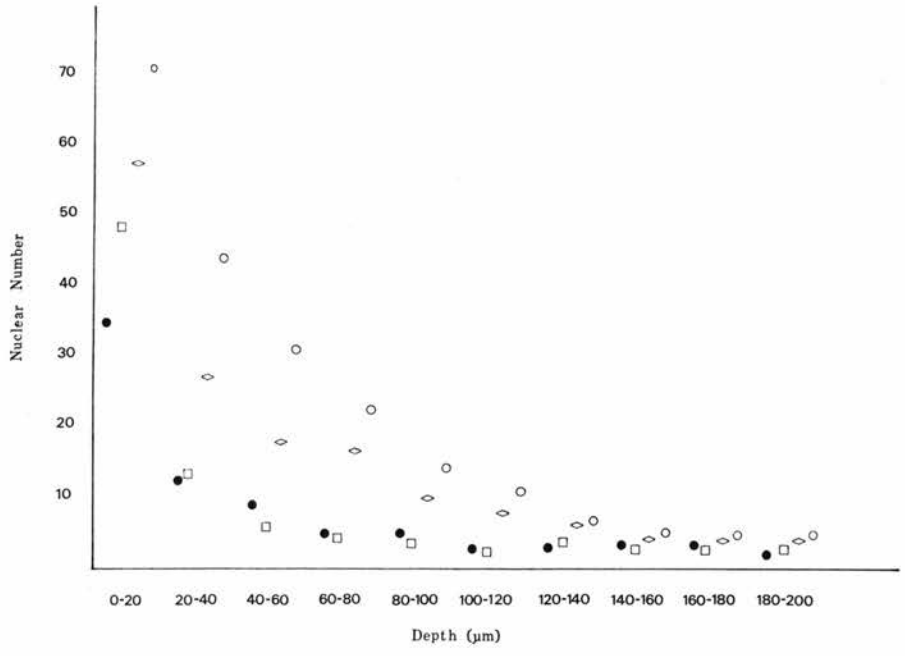
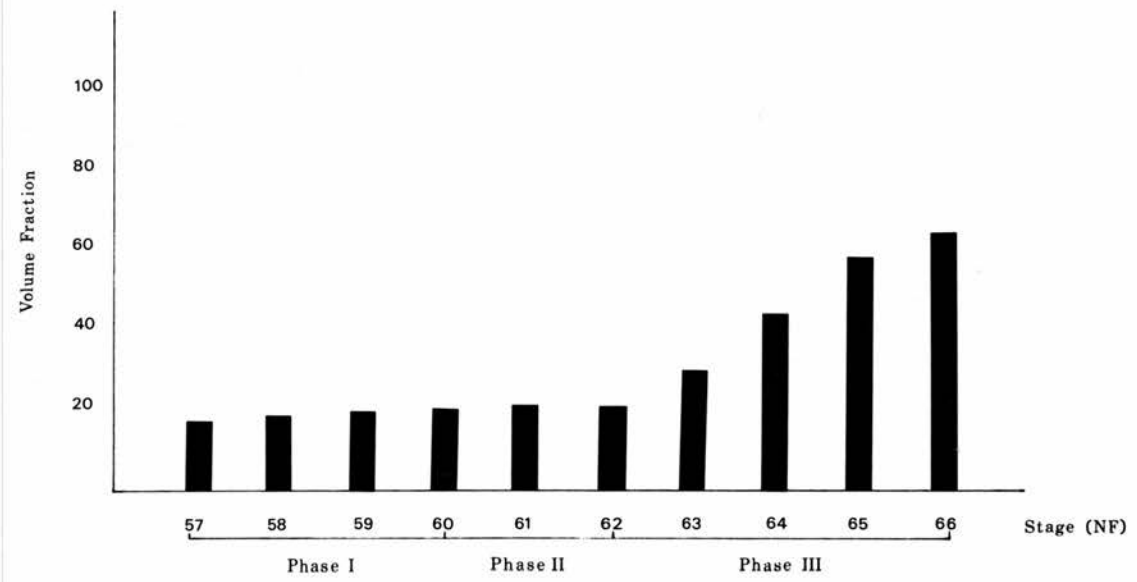

Nuclear Number
70
60
50
40
30
20
10
0-20
20-40
40-60
60-80
80-100
100-120
120-140
140-160
160-180
180-200
Depth (μm)

Fig 48

Histogram showing the volume fraction (%) of matrix present for each NF Stage during prometamorphosis and metamorphic climax, together with the developmental Phases described in Section III.


Volume Fraction
100
80
60
40
20
57
58
59
60
61
62
63
64
65
66
Stage (NF)
Phase I
Phase II
Phase III

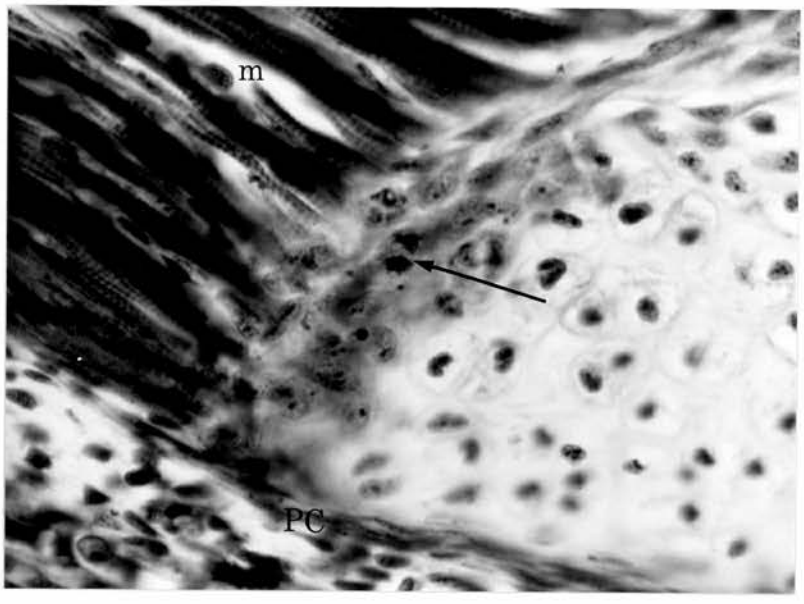
Figs 49a and b

Parts of the periphery of Meckel's cartilage at Stage 62NF showing mitotic figures. ⟶

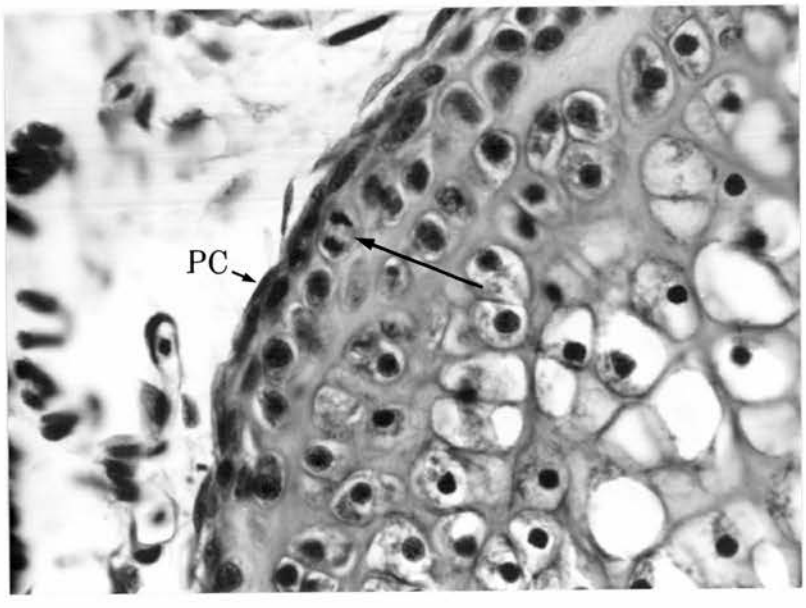
Horizontal sections x 160

PC perichondrium

m striated muscle fibres



a

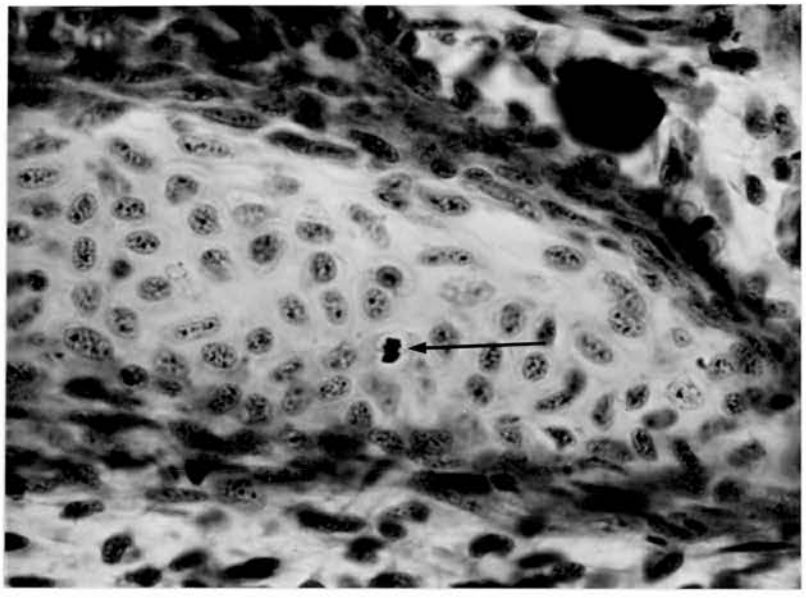


b

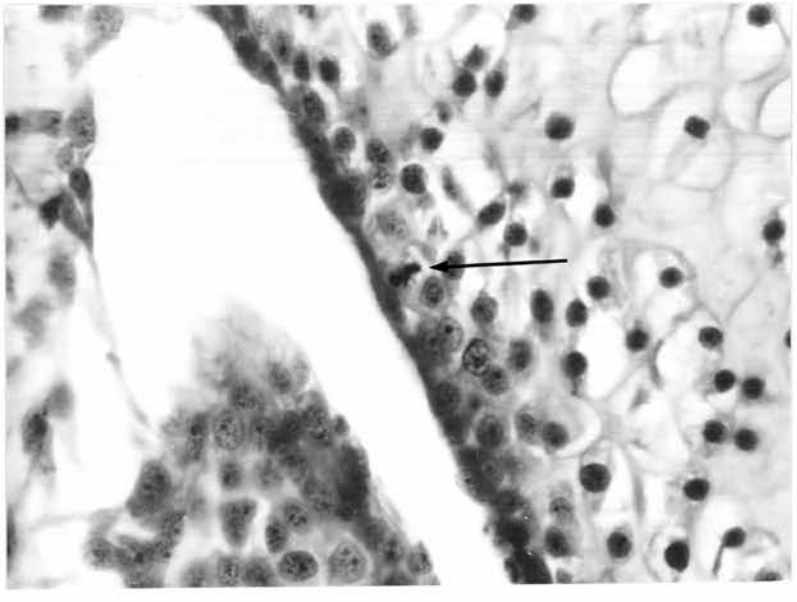
Figs 50a and b

Mitotic figures in the periphery of Meckel's cartilage following colchicine administration (1mg/L), for 24 hours at Stage 61NF. ⟶

Horizontal section x 160



a



b

Fig 51

Histogram showing numbers of mitotic figures ($x \frac{1}{100}$) at different depths within Meckel's cartilage.

0	-	20μm	Band A
20	-	40μm	Band B
40	-	60μm	Band C
60	-	80μm	Band D

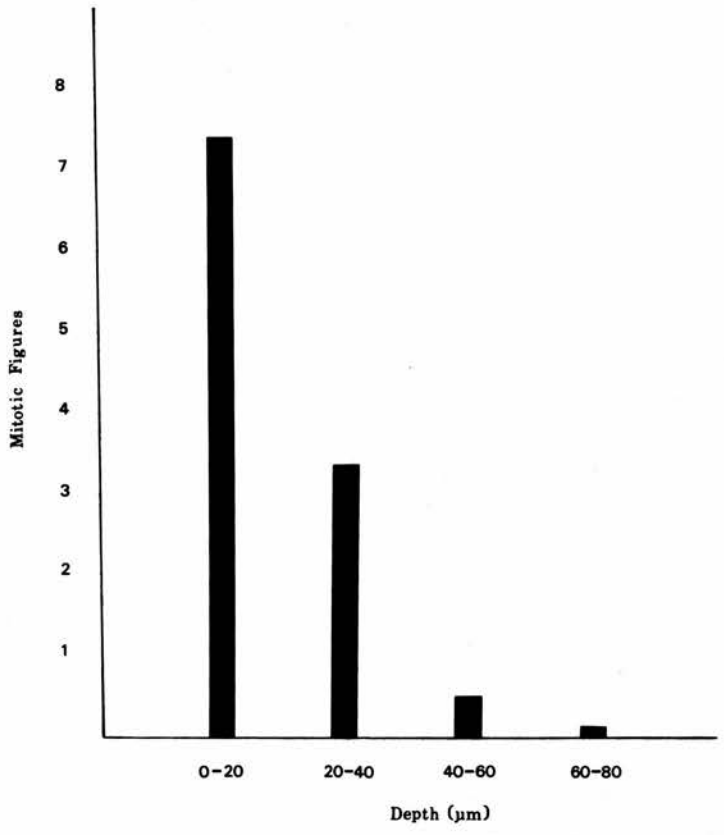

Mitotic Figures
8
7
6
5
4
3
2
1
0-20
20-40
40-60
60-80
Depth (μm)

Fig 52

Mitotic figures in epithelial cells following administration of colchicine (1mg/L) for 24 hours at Stage 61NF. ⟶

x 160

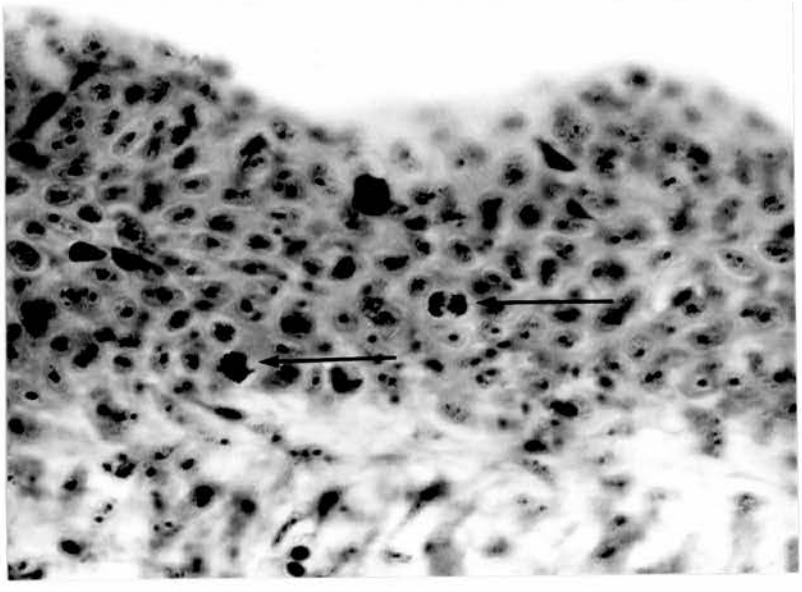


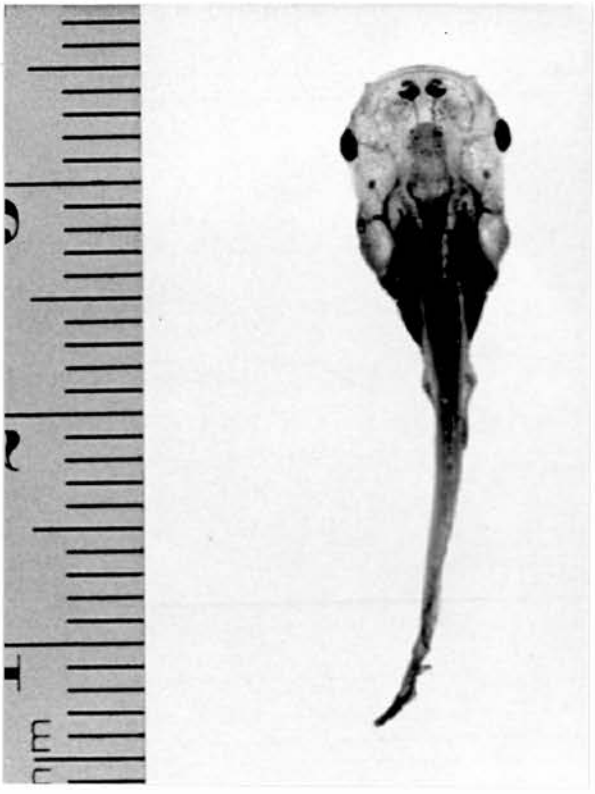
Fig 53

Tadpoles immersed in a solution of 1mg/ml of thyroid powder for 48 hours commencing at Stage 57NF.

A. Control

B. Experimental

Scale in millimetres



A

B

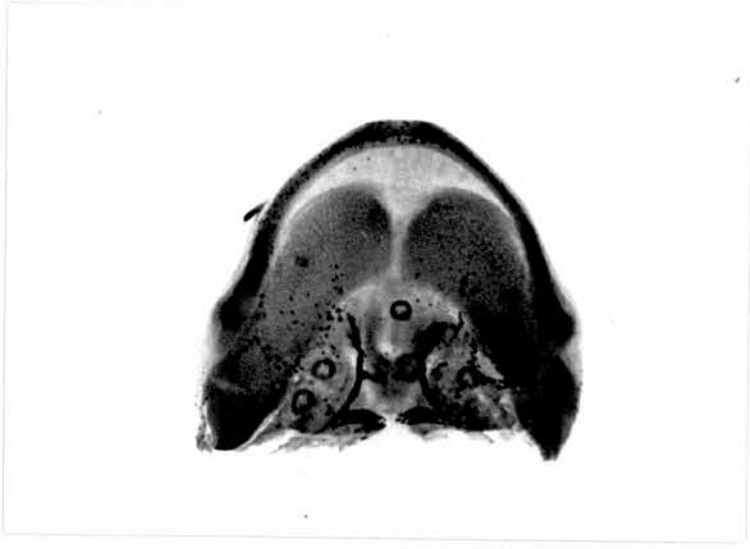
Fig 54

Lower jaw, following administration of 1mg/ml thyroid powder for 48 hours at Stage 57NF.

Cleared whole mount.

Fig 4 has been reproduced on the same page for comparison.

Scale = 1mm



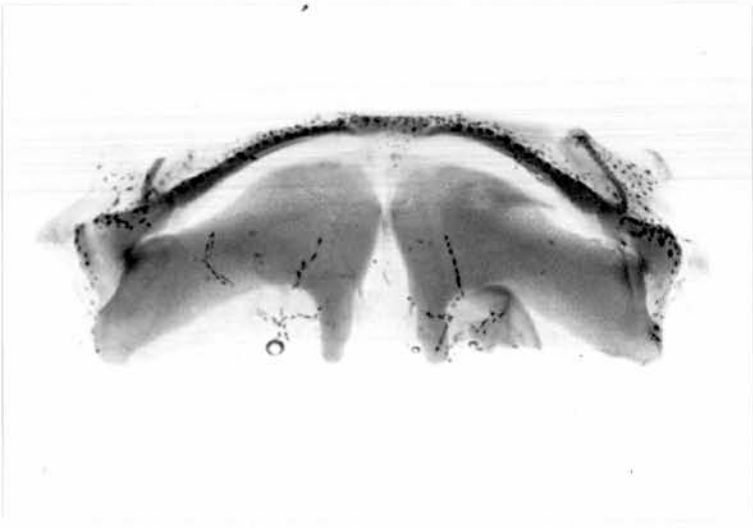
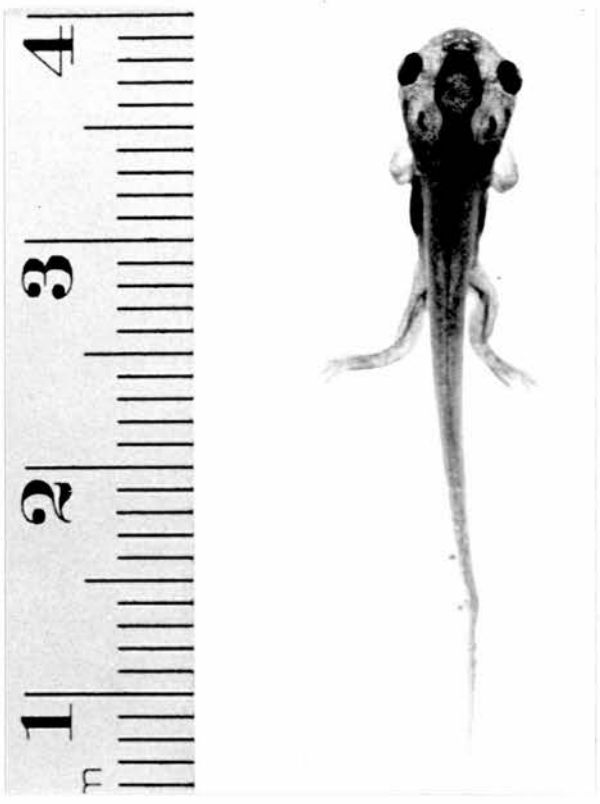


Fig 55

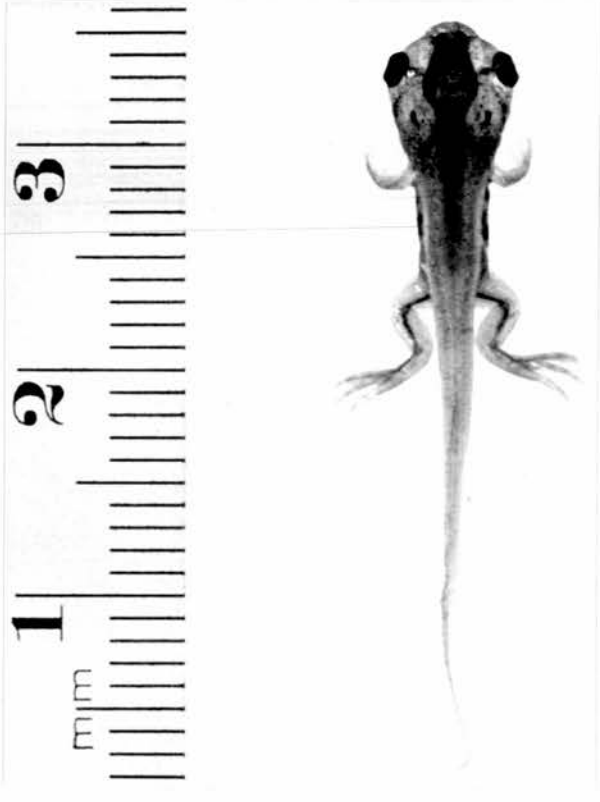
Tadpoles immersed in a solution of 1mg/ml of thyroid powder for 48 hours commencing at Stage 60NF.

A. Control

B. Experimental



A



B

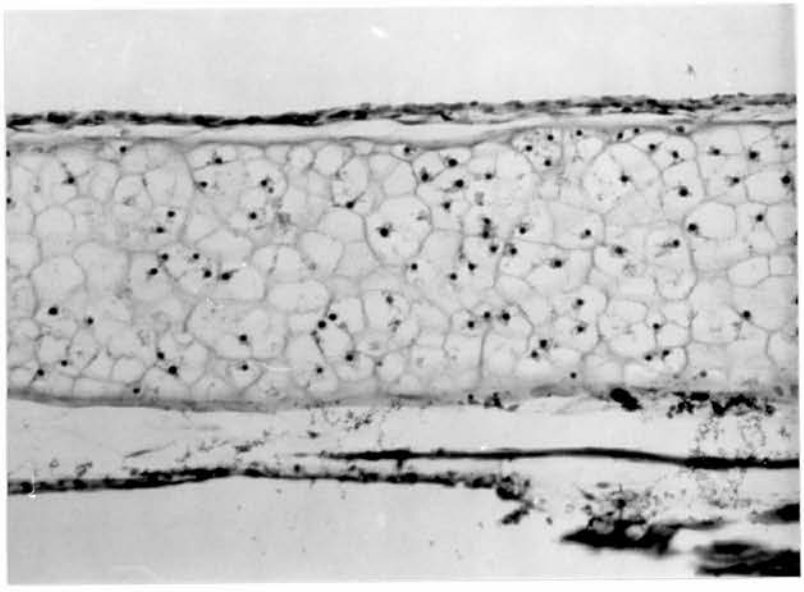
Fig 56

Parts of Meckel's cartilage following administration of lmg/ml thyroid powder for 48 hours commencing at Stage 57NF.

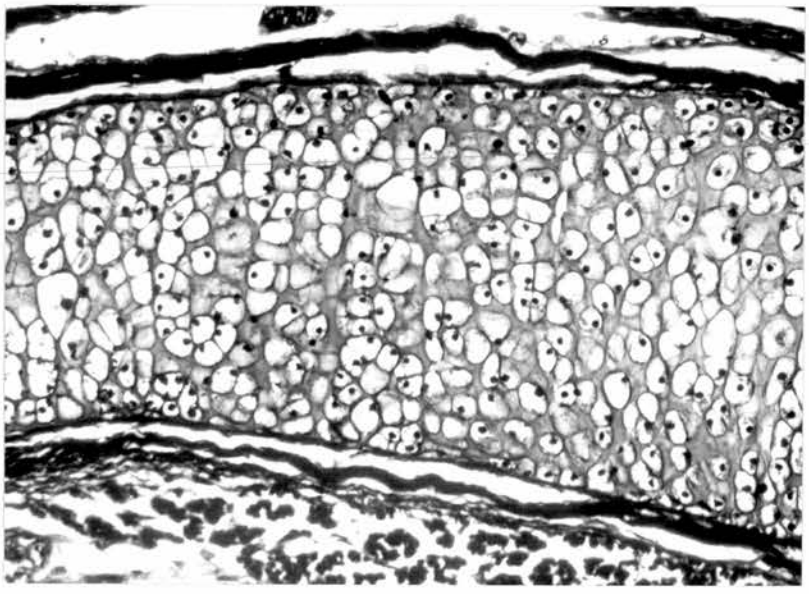
A. Control

B. Experimental

Horizontal sections x 64



A



B

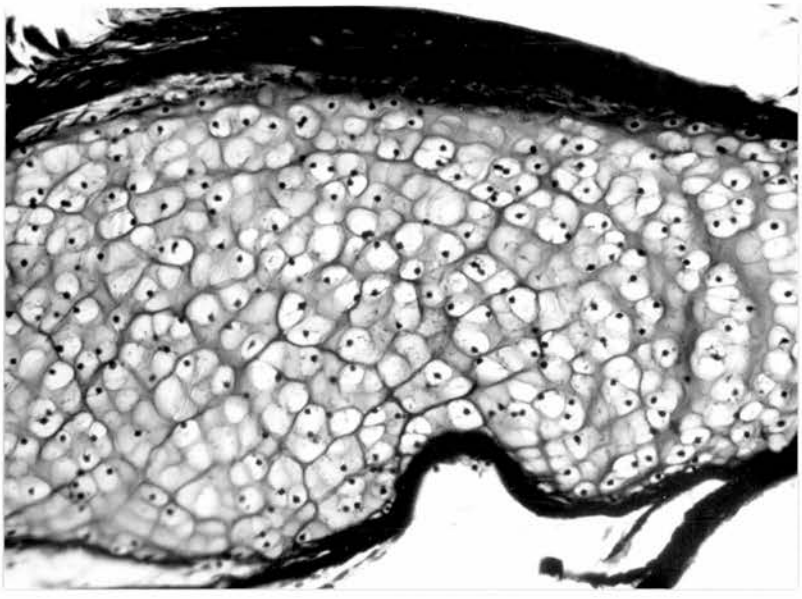
Fig 57

Parts of Meckel's cartilage following administration of 1mg/ml thyroid powder for 48 hours, commencing at Stage 60NF.

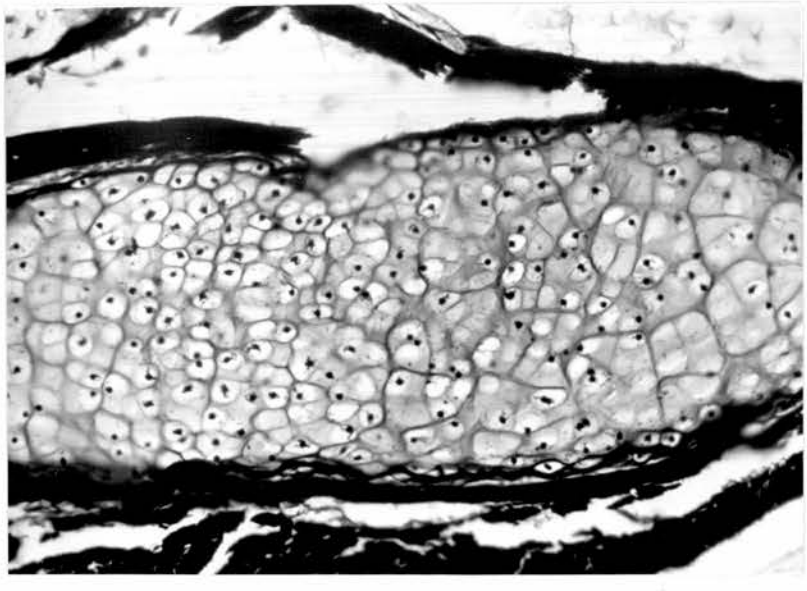
A. Control

B. Experimental

Horizontal sections x 64



A



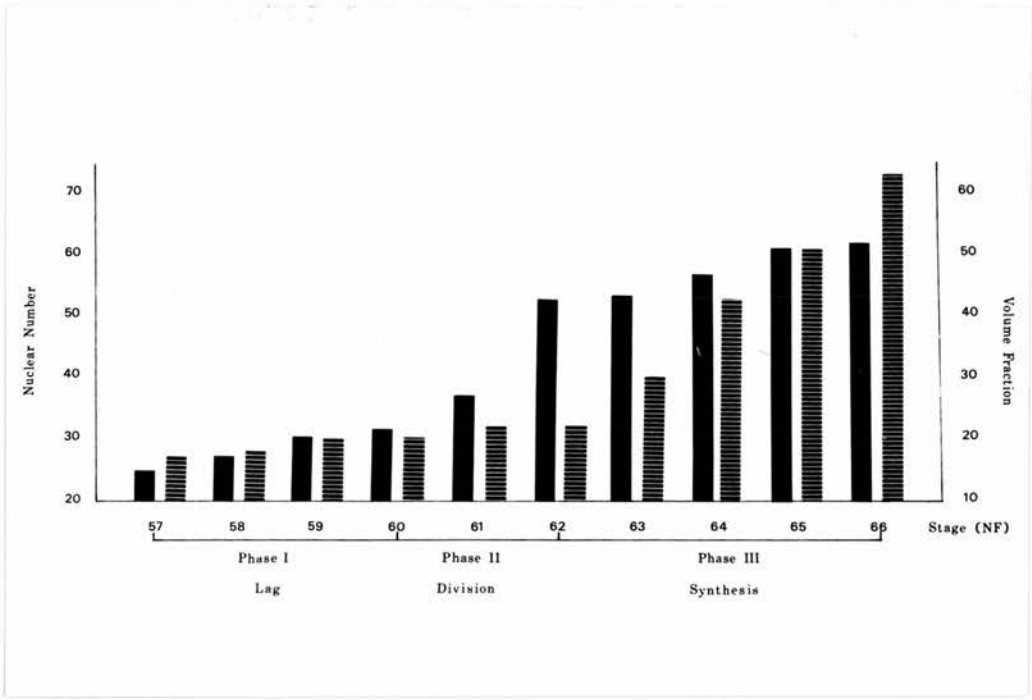
B

Fig 58

Combined histogram showing the corrected total nuclear number (x $\frac{1}{1000}$) and the volume fraction (%) of matrix present for each NF Stage during prometamorphosis and metamorphic climax, together with the developmental Phases.

Nuclear number 

Matrix volume fraction 


Nuclear Number
70
60
50
40
30
20
Volume Fraction
60
50
40
30
20
10
57
58
59
60
61
62
63
64
65
66
Stage (NF)
Phase I
Lag
Phase II
Division
Phase III
Synthesis